From Preeclampsia to Renal Disease

mechanistic studies

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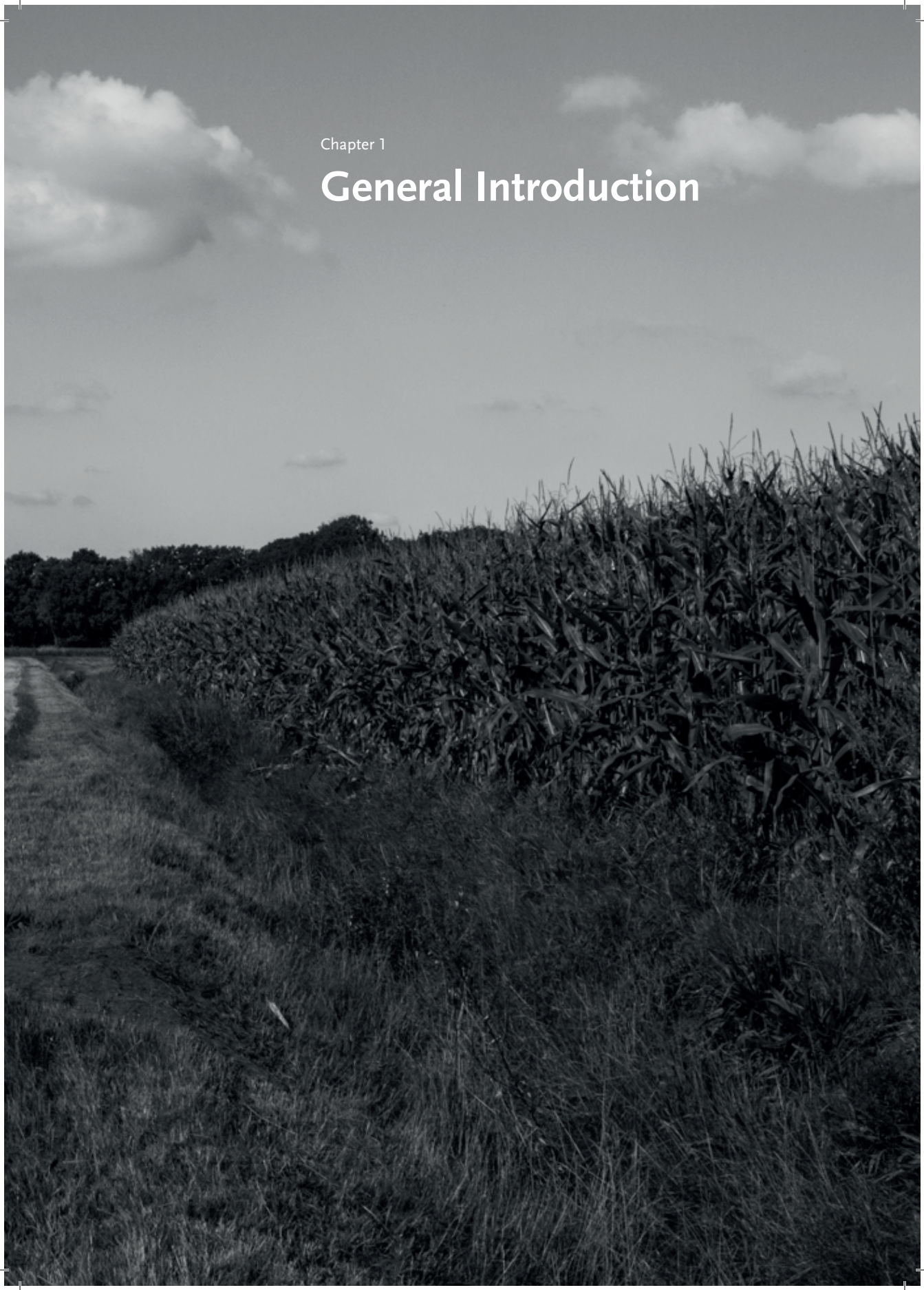

GENERAL INTRODUCTION

During pregnancy, the maternal hemodynamic system undergoes major changes in order to adapt to the pregnant condition. Blood volume and cardiac output increase, while the systemic vascular resistance decreases. This decrease in systemic vascular resistance is facilitated through changes in endothelial function and in activity of the renin-angiotensin aldosterone system (RAAS), and is clinically apparent from a slight decrease in blood pressure, mainly during the first and second trimester of pregnancy. In the kidney, vascular resistance also decreases substantially, with consequently a marked rise in renal blood flow and glomerular filtration rate (GFR).

The importance of these pregnancy-specific adaptations for a healthy course of pregnancy is highlighted by our current knowledge that maladaptation of the maternal hemodynamics to pregnancy is associated with pregnancy complications such as preeclampsia. Preeclampsia is characterized by hypertension and proteinuria in the second half of pregnancy, and has major impact on maternal and fetal health. To date, there is no real treatment option for preeclampsia apart from delivery, which usually results in resolution of the maternal signs and symptoms of preeclampsia within days.

In the last decade, it has become apparent that these women may not be completely cured from preeclampsia after delivery, as cohort studies report an increased risk for cardiovascular and renal disease later in life in these women. Whether the increased risk is a consequence of preeclampsia itself, or whether, alternatively, common (pre-pregnancy) risk factors contribute both to the development of preeclampsia and the increased long-term risk is still matter of debate. Also, the mechanisms behind this increased risk for cardiovascular and renal disease remain unknown. Since preeclampsia is characterized by vascular and hemodynamic maladaptation during pregnancy, it can be hypothesized that the maladaptive profile is not completely restored postpartum, thereby increasing the susceptibility to cardiovascular and renal disease in women with a history of preeclampsia. Elucidating the mechanisms involved in the increased cardiovascular and renal risk later in life in formerly preeclamptic women is important to understand why these women are more susceptible to cardiovascular and renal disease, and will guide the development of preventive strategies. In this thesis, therefore, we will study long-term renal function, RAAS, and endothelial function in women with a history of preeclampsia and in rats following experimental preeclampsia.

Preeclampsia

Preeclampsia, a pregnancy specific syndrome, complicates up to 6-8% of all pregnancies worldwide and is the leading cause of fetal and maternal morbidity and mortality [1, 2]. Preeclampsia is clinically characterized by de novo hypertension and proteinuria in the second half of pregnancy [3]. Preeclampsia can also occur superimposed upon chronic hypertension or pre-existing proteinuria. Preeclampsia can be classified by the gestational age at onset of the disease or by severity of the disease. Early-onset preeclampsia is defined as onset of preeclampsia before 34 weeks of gestation and severe preeclampsia is defined by blood pressure values >160mmHg systolic or >110mmHg diastolic [4, 5].

Preeclampsia is a systemic disorder that affects multiple maternal organs including the vascular bed, leading to generalized endothelial dysfunction, and the kidneys. Specific glomerular lesions are present in preeclampsia, named glomerular endotheliosis [6]. Therefore, preeclampsia can

be considered the most common glomerular disease worldwide [6]. Moreover, preeclampsia can affect the liver as part of the HELLP syndrome (Haemolysis Elevated Liver enzymes and Low Platelets) [3], or the brain, resulting in eclampsia. Eclampsia is the convulsive form of preeclampsia and affects 0.1% of all pregnancies (reviewed by Brezan, E et al. [2]). Although the pathophysiology of preeclampsia has been studied widely, the exact mechanism underlying the development of preeclampsia remains unclear. The prevailing hypothesis states that preeclampsia can be considered a multifactorial two-stage condition, involving the immune system, angiogenic factors, and the endothelium, with the placenta being the origin, that triggers the multi-system abnormalities that characterize preeclampsia [7-9].

Unfortunately, there is no treatment option to cure a woman from preeclampsia apart from termination of pregnancy; and thereby delivery of the placenta [10]. This often results in premature birth. Several options have been studied as potential preventive treatment for preeclampsia, including anti-oxidants [11], fish oil [11], and low sodium diet [12], with however disappointing results. Current studies focus on restoring the anti-angiogenic balance and deficiency in nitric oxide, and treatment with L-arginine, statins, relaxin, and other therapies to restore angiogenic factors are under investigation [2, 13, 14]. Aspirin and calcium supplementation (in populations where there is inadequate intake) have small beneficial effects in preventing preeclampsia in high-risk populations and may be effective when started early in gestation [10, 15].

Pathophysiology of preeclampsia

Current understanding of the syndrome describes preeclampsia as a two-stage syndrome with the placenta as the organ of origin [7, 16]. Although in both early and late-onset preeclampsia, placental dysfunction is involved, the mechanisms of placental dysfunction, and its role in the pathophysiology of preeclampsia seems to be different [8]. During early-onset preeclampsia impaired placentation plays a key role in the pathogenesis of preeclampsia, where in late-onset preeclampsia placental dysfunction is not due to impaired placentation, but may be related to its functional limit at the end of pregnancy. The placental dysfunction (stage 1) is then followed by the release of factors by the diseased placenta into the maternal circulation (stage 2), inducing widespread endothelial dysfunction [17] and inflammation [18] resulting in the classic manifestation of the syndrome. In late-onset preeclampsia, pre-pregnancy maternal endothelial health may also play an important role [19].

During normal development of the placenta, trophoblasts migrate through the decidua and part of the myometrium to invade the spiral arteries and play a role in the remodelling of the wall of the spiral artery such that the vascular wall is broken-down [20]. Due to the process of spiral artery remodelling, the spiral arteries transform into large capacitance vessels of low resistance and sufficient blood supply to the placenta and growing foetus is thereby guaranteed [20, 21]. During the first stage of early-onset preeclampsia, the invasion of the trophoblasts is limited, mainly in the myometrium, resulting in incomplete remodelled spiral arteries, leading to small muscular spiral arteries [22-24]. Due to this incomplete spiral artery remodelling, maternal blood will enter the intervillous space intermittently and with increased velocity, resulting in villous damage, ischaemia reperfusion injury, and oxidative stress of the chorionic villi [25].

The maternal reaction to this poor placentation is the so-called second stage of preeclampsia. Over the past years, several studies report that in the preeclamptic placenta, the syncytiotrophoblast produces and secretes several factors, mainly pro-inflammatory and anti-angiogenic factors [26-28]. These factors are then via the intervillous space spread into the maternal circulation [26, 29] and lead to a generalized activation of the inflammatory system, endothelial dysfunction and culminate into the symptoms of preeclampsia.

Late-onset preeclampsia is suggested to be a maternal syndrome with no evidence of decreased spiral artery remodelling [3, 30, 31]. However, it has been hypothesized recently, that late-onset preeclampsia can be considered as a process of restricted intervillous perfusion near term. Near term, placental growth reaches its functional limit restricting intervillous perfusion. This leads to disturbed placental perfusion and thereby oxidative stress in e.g. (syncytio)trophoblast cells [8]. As in early-onset preeclampsia, these stressed syncytiotrophoblast cells then release several factors into the maternal circulation inducing the clinical stage (stage 2) of preeclampsia. However, several pre-existing maternal disorders, i.e. hypertension, obesity, and diabetes mellitus, have also been associated with the occurrence of late-onset preeclampsia (reviewed by Ness, R.B. et al. [30]). In these women, endothelial dysfunction is present pre-pregnancy, and it is thought that together with the increased systemic inflammatory status seen in healthy pregnancy, this contributes to the clinical symptoms of preeclampsia [19].

Development of clinical signs of preeclampsia

Role of the inflammatory response

During healthy pregnancy, circulating inflammatory cells, i.e. granulocytes and monocytes, become activated [32]. During pregnancy, circulating leukocytes come into direct contact with the syncytiotrophoblasts in the intervillous space. This may result in activation of inflammatory cells [33]. Moreover, the healthy placenta sheds syncytiotrophoblast micro- and nanoparticles and other factors into the maternal circulation [34-37] and these particles and factors are thought to interact with the maternal immune system thereby stimulating the inflammatory response in pregnancy [38-40]. This activation of maternal inflammatory cells is even more pronounced during preeclampsia [40, 41]. Various factors, partly corresponding to those found in normal pregnancy, produced by the oxidatively stressed placenta, such as syncytiotrophoblast micro- and nanoparticles [35, 41], pro-inflammatory cytokines [42], chemokines [43], and ATP [44] are held responsible for the marked activation of the inflammatory cells in preeclampsia. Activation of these inflammatory cells together with endothelial dysfunction have widespread consequences that are characteristic for preeclampsia [7, 45].

Role of angiogenic factors

In normal pregnancy, pro-angiogenic factors (vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)) are balanced with anti-angiogenic factors (soluble Fms-like tyrokinase 1 (sFlt-1), soluble endoglin (sEng), and reactive oxygen species (ROS)) [46, 47]. During preeclampsia, this balance is disrupted; high levels of sFlt1 and sEng were found [27, 48], together with low levels of VEGF and PlGF [27]. The high circulating concentrations of anti-angiogenic proteins are assumed to

contribute to the stage 2 manifestation of preeclampsia. sFlt1 induces endothelial dysfunction by inhibiting VEGF signalling [49]. This is particularly true in endothelial cells that are fenestrated and have constitutive expression of VEGF, such as those that reside in the glomerulus of the kidney [50]. Inhibition of VEGF not only impairs angiogenesis, but might also impair endothelium-dependent vasodilation, contributing to a rise in blood pressure [51].

Role of maternal hemodynamics

To secure a healthy pregnancy, the maternal hemodynamic system has to adjust to the pregnant condition [52]. Changes in the endocrine environment of pregnant women lead to a decrease in systemic vascular resistance [53, 54] accompanied by a decrease in arterial stiffness [55, 56]. This decrease in systemic vascular resistance [57] enlarges the vascular capacity mimicking a state of under filling [58, 59]. Subsequently, plasma volume increases about 40%, thereby ensuring sufficient blood supply and oxygen and nutrition supply to the placenta and fetus [52]. In contrast, preeclampsia is characterized by a relative increased vascular resistance and arterial stiffness as compared to healthy pregnant women [60-63] and in concordance with this increase, the rise in plasma volume is diminished in preeclampsia [64, 65]. Adaptations of the RAAS [66], of the endothelial cells [67], and of the stiffness of the vascular wall [55] are all suggested to be involved in the maternal hemodynamic adaptations to pregnancy, while impairments in these adaptive mechanisms are assumed to contribute to the development of the maternal syndrome of preeclampsia. In the next paragraphs, the respective roles of the RAAS, endothelial function, and of arterial stiffness in the regulation of vascular tone are described in more detail.

Renin-angiotensin aldosterone system

Figure 1 represents a schematic and simplified overview of the RAAS. Activation of the RAAS occurs mainly by sympathetic activation, volume depletion, and hypotension. Renin stimulates the conversion from angiotensinogen (produced by the liver) to angiotensin I (ang I), which is not biologically active. Ang I is converted to angiotensin II (ang II) by angiotensin-converting enzyme (ACE). Ang II is the main effector hormone of the RAAS and induces vasoconstriction in the systemic and renal vascular bed [68] by binding to the ang II type I receptor (AT1-R) [69]. In addition, binding of ang II to the ang II type 2 receptor (AT2-R) leads to vasodilation [70] thereby opposing the effect of the AT1-R. Furthermore, ang II stimulates aldosterone release from the adrenal cortex [53]. Next to the classical ACE/ang II/AT1-R axis, a counterbalancing arm has been identified within the RAAS, consisting of angiotensin converting enzyme 2 (ACE2), angiotensin 1-7 and its receptors (Mas receptor and AT2-R) [53, 70].

The RAAS becomes activated during healthy pregnancy as response to the decrease in systemic vascular resistance [53, 71] and increased oestrogen levels [72]. Although activation of the RAAS leads to increased levels of renin and angiotensinogen, and thereby increased ang II levels [73, 74], healthy pregnant women are less sensitive to the vasoconstrictor actions of ang II compared to non-pregnant women [75]. Counteracting effects of other vasodilators and/or a decrease in AT1-R density are suggested mechanisms involved in this decreased sensitivity [53, 76, 77]. This decreased vascular

responsiveness to ang II contributes to the pregnancy-induced decrease in systemic vascular resistance.

During preeclampsia, plasma volume expansion and the decrease in systemic vascular resistance are less pronounced. In response, most circulating components of the RAAS, such as ang II, are lower during preeclampsia as compared to a healthy pregnancy [78]. However, importantly, the diminished ang II sensitivity seen in healthy pregnancy is blunted in preeclampsia [79-81]. Since ang II is an important vasoconstrictor agent, this non-adaptation of ang II sensitivity might play an important role in hypertension during preeclampsia [82]. The exact mechanisms behind the increased ang II sensitivity are unknown but possibly altered placental and vascular AT1-R expression are involved [83-85]. Other mechanisms like decreased angiotensin 1-7 expression [86], the presence of AT1-R autoantibodies [83, 87, 88], and decreased hemopexin activity [89] could also be involved.

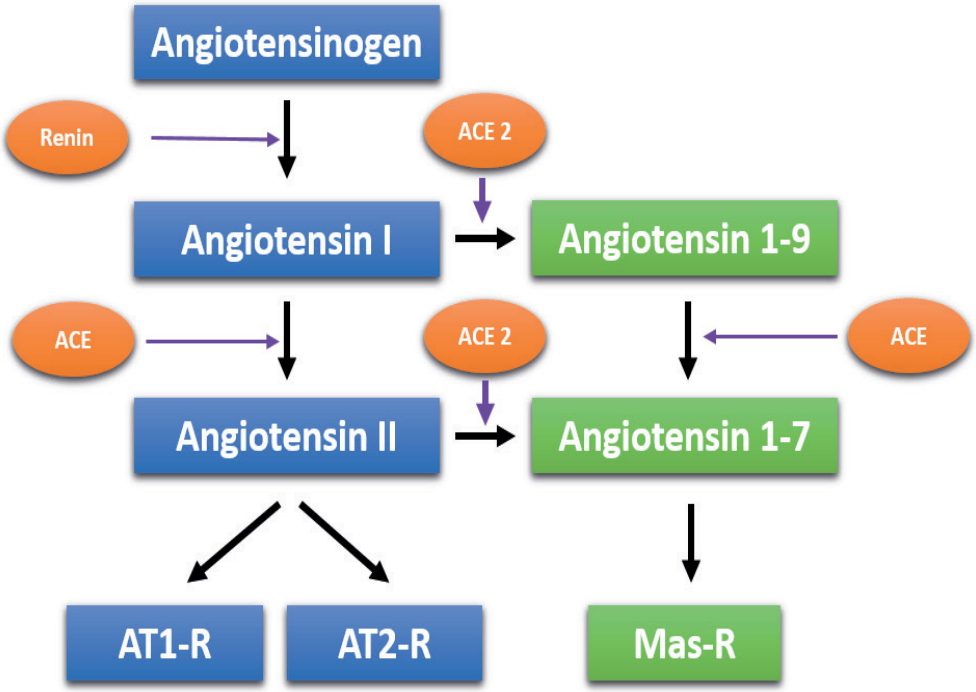

Figure 1. Simplified overview of the renin-angiotensin aldosterone system.

ACE: angiotensin converting enzyme, AT1-R: angiotensin II type 1 receptor, AT2-R: angiotensin II type 2 receptor. Adapted from Conti S et al. Hypertension 2012;60:878-883

Endothelial function

The endothelium, by production of vasoactive factors, is important in the regulation of the vascular tone [90, 91]. Important vasoactive factors are nitric oxide (NO), the prostanoids, and unidentified endothelium-derived hyperpolarizing factor(s) (EDHF) [92]. NO is a potent vasodilator [93] and is formed by the conversion of L-arginine by three different NO synthase (NOS) isoforms; endothelial NOS, inducible NOS, and neuronal NOS [93]. Prostanoids derive from arachidonic acid and via conversion by cyclooxygenase, prostaglandins (mainly prostacyclin and PGE_2) and thromboxane [94] are produced. In vascular tone regulation, prostacyclin and PGE_2 induce vasodilation and thromboxane induces vasoconstriction [95-97]. Next to NO and prostanoids, there is a third group of endothelial-derived factors, which are able to induce vasodilation, collectively named EDHF [98, 99]. EDHF is associated with hyperpolarisation of the smooth muscle cells via influencing the influx of potassium ions [100]. One member of the EDHF group is hydrogen sulfide (H_2S) [101] which is suggested to enhance vasodilation [102].

Changes in the production of these endothelium-derived vasoactive factors may account for the pregnancy-related decrease in systemic vascular resistance. During pregnancy, NO production and NO bioactivity are increased as compared to non-pregnant women [103, 104]. In addition, eNOS protein expression is increased in human uterine arteries during pregnancy [105]. Also, the prostaglandin pathway may be important in the regulation of the vascular tone during pregnancy [106], since increased production of prostacyclin has been found during pregnancy [107-109]. The contribution of EDHF in endothelium-dependent vasodilation has also been suggested to be increased during healthy pregnancy [110-112]. H_2S has recently been linked with pregnancy, since in the placenta [113], the enzymes involved in H_2S production are active and able to produce H_2S [114].

Preeclampsia is characterized by endothelial dysfunction whereby impaired regulation and secretion of the endothelium-derived vasoactive factors occurs, thereby increasing the systemic vascular resistance [115]. NO bioavailability is decreased during preeclampsia [93] and the balance between the prostanoids, prostacyclin and thromboxane, is towards increased thromboxane levels, leading to increased vasoconstriction [96, 109, 116-123]. The contribution of EDHF is suggested to be reduced in preeclampsia [110, 124] and down regulation of the enzymes involved in H_2S production has been shown in the placenta [113, 125, 126].

Arterial stiffness

Arterial stiffness, defined as a reduced capacity of an artery to dilate or contract in response to pressure changes [127], is another important modulator of vascular tone. It can be assessed non-invasively by analysing the peripheral arterial pulse waveform using indirect measures such as pulse wave analysis (PWA) [128] or pulse wave velocity (PWV) [129]. Arterial stiffness increases with aging and in several disease conditions, such as diabetes mellitus, atherosclerosis, hypertension, and chronic kidney disease [130-133]. Moreover, an increased arterial stiffness is an independent risk factor for cardiovascular diseases [134]. Although arterial stiffness is highly associated with structural changes of the arterial wall [135], it is also known that vascular tone can reversibly affect arterial stiffness (reviewed by Wilkinson, I.B. et al. [136]). Therefore, mechanisms that regulate vascular tone, i.e. endothelial

function, can also affect arterial stiffness in the short term [131, 137].

During pregnancy, starting around 5 weeks, arterial stiffness is decreased [55, 138-140]. In contrast, in women who develop preeclampsia, an increase in arterial stiffness as compared to control pregnant women is shown already in the first trimester of pregnancy [60-63]. The mechanism behind these changes in arterial stiffness during pregnancy and preeclampsia remains to be elucidated. In healthy pregnancy, the increased oestrogen level causing relaxation of the muscular layer of the vessel wall is suggested to play a role in the decrease in arterial stiffness [141]. In addition, the vascular filling state, the decreased blood pressure, and the rise in heart rate may be involved in the pregnancy-induced decrease in vascular stiffness [142]. Also the mechanism of increased arterial stiffness before and during preeclampsia remains to be established. It may however be suggested that endothelial dysfunction, which is prominent in preeclampsia [17], may result in impaired release of endothelial-derived vasoactive factors, thereby enhancing arterial stiffness [137].

Animal models of preeclampsia

Preeclampsia occurs only in humans and, rarely, in higher apes [143]. Therefore, much effort has been put into the development of animal models, which ideally are pregnancy specific and mimic a majority of the spectrum of the biochemical and clinical features of human preeclampsia. Widely used models, mainly in mice and rats, include inflammatory models with low-dose endotoxin (lipopolysaccharides; LPS) [144], ATP [145] or IL-6 [146]. Also non-inflammatory models are widely used, i.e. overexpressing sFlt-1 [27], decreased uteroplacental perfusion by clipping the aorta or uterine arteries [147, 148], chronic NOS inhibition [149, 150], and RAAS overactivity [151] or genetic adjustments [152-154].

Over the years, our research group has gained experience in working with the low-dose endotoxin rat model for preeclampsia. This model will be used in the current thesis and has the following characteristics. It is based on infusion of a low-dose endotoxin on day 14 of pregnancy [144]. In response to this infusion, a generalized low-grade inflammatory response, including endothelial cell activation, develops [155]. This response is considered the underlying mechanism of the development of hypertension and proteinuria in pregnant rats infused with endotoxin [156-160].

As pointed out earlier, both the RAAS, via ang II sensitivity, and endothelial dysfunction via impairing the endothelium-dependent relaxation, are thought to play an important role in preeclampsia, but so far, these have not yet been well characterized in this model.

Renal function in pregnancy and preeclampsia

During healthy pregnancy glomerular hyperfiltration up to 40-60% above the habitual level of renal function occurs in the second half of pregnancy [161] accompanied by an increased effective renal plasma flow (ERPF) [162]. The relationship between the GFR and ERPF changes throughout pregnancy leading to alterations in filtration fraction (FF=GFR/ERPF) as pregnancy advances, i.e. lower FF during first trimester to higher FF in third trimester as compared to non-pregnant women [163]. At term, GFR is still 40% higher than in non-pregnant women and then declines to normal, non-pregnant levels, one month after delivery [164]. All values (ERPF, GFR, and FF), normalize 4-6 weeks after delivery [165].

These functional changes in renal hemodynamics are different during preeclampsia. In women with preeclampsia, GFR is significantly lower than in healthy pregnant women [166], without differences in ERPF, resulting in a decreased FF during preeclampsia [167]. The mechanism of the decreased GFR during preeclampsia has not been elucidated. Changes can be functional, due to altered hemodynamics, or due to secondary effects of structural renal changes, i.e. high blood pressure and albuminuria [6, 166, 168], or due to primary renal changes, such as podocyte alterations [169]. Histologically, glomerular endotheliosis is seen [6], characterized by fibrin deposition, endothelial swelling and detachment, reduced density and size of endothelial fenestrae, thickening of the glomerular basement membrane, and loss of capillary space [6, 167, 170]. The finding that women with chronic kidney disease experience an increased risk for developing preeclampsia when pregnant [171], highlights the importance of accurate renal hemodynamic adaptations to pregnancy.

Long-term consequences of preeclampsia – cardiovascular and renal perspective

For a long time, preeclampsia was thought to be a reversible syndrome, with full recovery after pregnancy. However, over the past years epidemiologic studies have shown consistently that preeclampsia carries an increased risk for premature cardiovascular and renal disease in later life. Women with a history of preeclampsia experience a 3-4 fold increased risk for long-term cardiovascular disease [172] and approximately a 5-12 fold increased risk for end stage renal disease [173-175].

Whether renal function is affected in formerly preeclamptic women is debated. Current small studies (mostly up-to 10 years post-partum) report no difference or just subtle differences in renal function and renal hemodynamics after preeclampsia. A high normal estimated GFR (eGFR) has been reported but other studies showed no differences in renal function [176-178]. However, in studies that found altered renal parameters in formerly preeclamptic women, co-morbidity, i.e. hypertension, was present. This may well have caused these alterations [179, 180]. The persistence of proteinuria postpartum is subject of debate as well. Proteinuria has been reported to persist in the postpartum period in a considerable number of formerly preeclamptic women. A meta-analysis showed that the occurrence of micro-albuminuria is as high as 31% in formerly preeclamptic women versus 7% in controls [177]. However, individual retrospective studies, report lower incidences of proteinuria in formerly preeclamptic women [176, 181, 182]. Both persistent renal hemodynamic alterations and persistent proteinuria following preeclampsia can potentially be involved as pathophysiological mechanisms underlying the increased susceptibility to cardiovascular and renal disease later in life.

The underlying pathways leading to cardiovascular and renal disease in women with a history of preeclampsia remain to be fully elucidated. It is generally assumed that the increased risk results from the presence of pre-existing cardiovascular risk factors in combination with preeclampsia induced disturbances that persist postpartum or develop after a preeclamptic pregnancy [183-186]. To differentiate between pre-pregnancy cardiovascular (risk) factors and the effect of preeclampsia itself on the future cardiovascular and renal health, ideally long-term follow-up studies starting pre-pregnancy need to be performed. However, with the relatively low incidence of preeclampsia, these studies are almost impossible to perform. Therefore, to discriminate between pre-pregnancy risk

factors and preeclampsia-induced impairments, studies focussing on the long-term consequences of preeclampsia, which only include currently healthy women following preeclampsia are needed. Also, animal models are needed, in which the animals can be studied before, throughout, and after an experimental preeclamptic pregnancy. The advantage of studying animal models, is that all animals have the same pre-pregnancy health.

As described above, plasma levels of ang II are decreased while the sensitivity for ang II is increased during preeclampsia. Persistent post-partum increased ang II sensitivity might potentially play a role in the increased cardiovascular and renal risk after preeclampsia [187, 188]. Moreover, arterial stiffness is suggested to be increased during preeclampsia and arterial stiffness has been identified as an independent risk factor for cardiovascular diseases [189-191]. It can be hypothesized that persistent post-partum increased ang II sensitivity and arterial stiffness may also be involved in the mechanism behind the increased cardiovascular and renal risk following preeclampsia [192, 193].

This thesis presents studies performed in women, without comorbidities, after preeclampsia and in an animal model for experimental preeclampsia, to investigate the possible role of renal function, the RAAS, endothelial function, and arterial stiffness as mechanisms in the increased susceptibility to cardiovascular and renal disease after preeclampsia.

Aim of this thesis

The aim of this thesis is to gain more insight into the mechanisms behind the increased late risk for cardiovascular and renal diseases in women with a history of preeclampsia. We hypothesized that changes in the RAAS and maternal (renal) hemodynamics (including endothelial function) are involved. These changes could be pre-existing, and hence contribute to the development of both preeclampsia and cardiovascular and renal disease, and/or could be induced by preeclampsia itself. To differentiate between the contribution of comorbid conditions and of preeclampsia itself, women with a history of preeclampsia without comorbid conditions were studied, in parallel with studies in an animal model of experimental preeclampsia (i.e. the low-dose endotoxin infused pregnant rat). To this purpose, first the changes in the RAAS and endothelium during experimental preeclampsia in this animal model were characterized. This was done in **Chapter 2** of this thesis. Thereafter, from **Chapter 3 to Chapter 7**, we focussed on the long-term renal risk after preeclampsia in humans and in the animal model, and studied potential mechanisms involved in the susceptibility of the increased cardiovascular and renal risk following (experimental) preeclampsia.

Animal models for preeclampsia are essential to study the pathophysiology of this syndrome. Ideally, the phenotype of experimental preeclampsia closely resembles the human situation. Since we aim to study the role of the RAAS and endothelial function in the increased risk for cardiovascular and renal diseases in later life, we aimed to select an animal model that closely resembles preeclampsia in these respects. However, whether or not these features were present in the low-dose endotoxin infused pregnant rat was unknown. Therefore, we first characterized the changes in the RAAS and endothelial function in this model.

- In **Chapter 2**, we studied whether the endotoxin rat model for preeclampsia is characterized by increased ang II sensitivity and endothelial dysfunction

Then, from **Chapter 3 to Chapter 7** of this thesis, we focus on the renal risk and mechanisms involved in this renal risk in women after preeclampsia. To rule out effects of common pre-pregnancy cardiovascular risk factors, we included healthy, normotensive women, selected for absence of comorbidity and without signs of underlying cardiovascular diseases. In addition, since sodium status is a well-known modulator of RAAS activity and affects endothelial function, the prospective studies were performed with a standardized sodium intake. We also studied healthy pregnant rats in which experimental preeclampsia was induced by low-dose endotoxin infusion during pregnancy.

- In **Chapter 3**, we review the proposed mechanisms behind the increased risk for renal disease in formerly preeclamptic women

- In **Chapter 4**, long-term follow-up of renal function data collected in the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) study were used to study the long-term course of post-partum renal function in a large cohort of women with self-reported hypertensive disorders of pregnancy and in women with a history of pregnancy induced hypertension, HELLP or preeclampsia 10-years postpartum
- In **Chapter 5**, we studied renal hemodynamics in healthy formerly preeclamptic women as a potential mechanism behind the increased risk for renal disease later in life in these women
- In **Chapter 6**, we studied the ang II sensitivity in healthy formerly preeclamptic women and in formerly experimental preeclamptic rats as a potential mechanism behind the increased risk for cardiovascular and renal disease later in life in these women
- In **Chapter 7**, we studied arterial stiffness and markers for endothelial dysfunction in healthy formerly preeclamptic women, in relation to sodium intake

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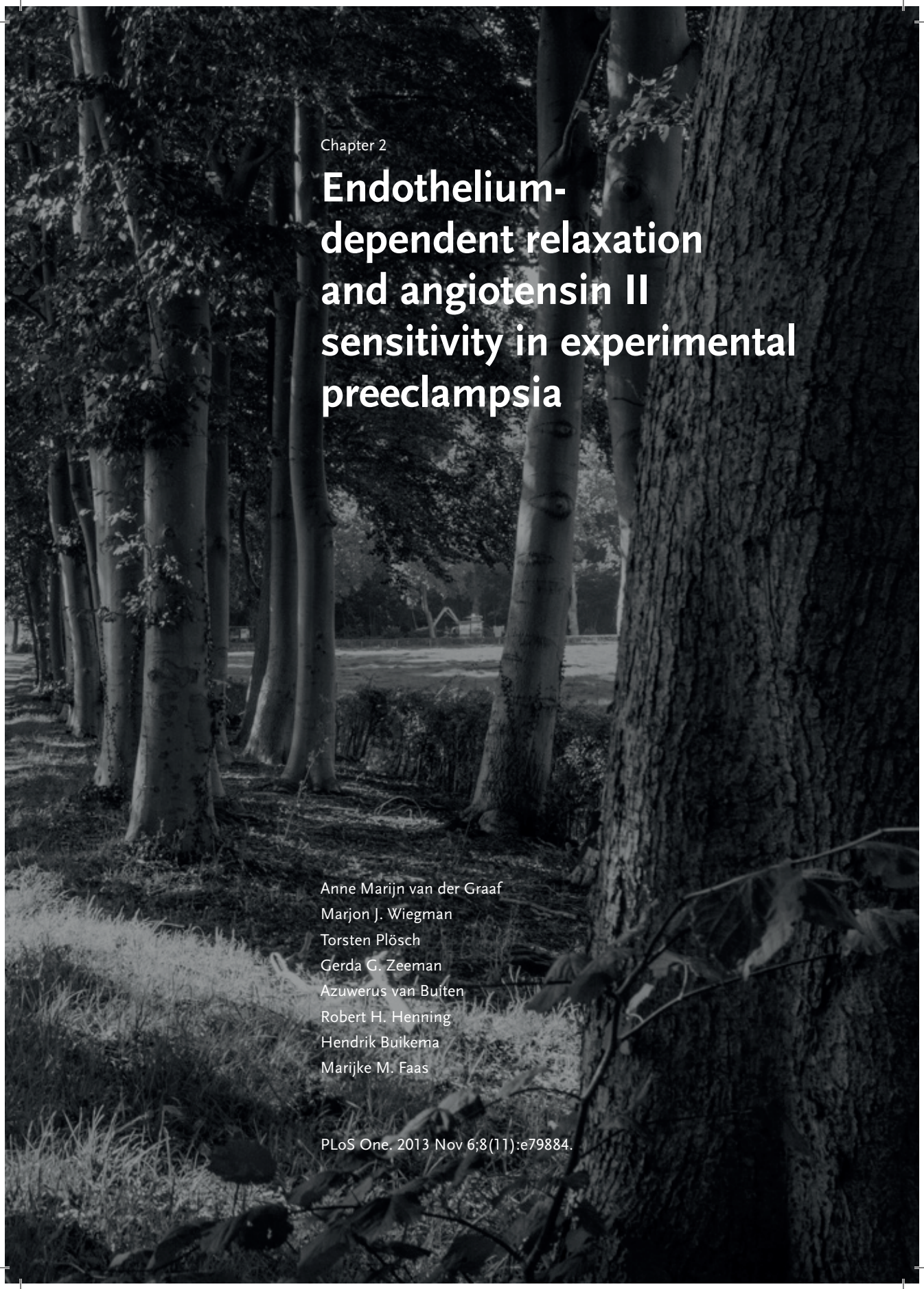

Chapter 2

Endothelium-dependent relaxation and angiotensin II sensitivity in experimental preeclampsia

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ABSTRACT

Objective

We investigated endothelial dysfunction and the role of angiotensin (Ang)-II type I (AT1-R) and type II (AT2-R) receptor in the changes in the Ang-II sensitivity in experimental preeclampsia in the rat.

Methods

Aortic rings were isolated from low dose lipopolysaccharide (LPS) infused pregnant rats (experimental preeclampsia; n=9), saline-infused pregnant rats (n=8), and saline (n=8) and LPS (n=8) infused non-pregnant rats. Endothelium-dependent acetylcholine-mediated relaxation was studied in phenylephrine-preconstricted aortic rings in the presence of vehicle, N^{G}-nitro-L-arginine methyl ester and/or indomethacin. To evaluate the role for AT1-R and AT2-R in Ang-II sensitivity, full concentration response curves were obtained for Ang-II in the presence of losartan or PD-123319. mRNA expression of the AT1-R and AT2-R, eNOS and iNOS, COX1 and COX2 in aorta were evaluated using real-time RT-PCR.

Results

The role of vasodilator prostaglandins in the aorta was increased and the role of endothelium-derived hyperpolarizing factor and response of the AT1-R and AT2-R to Ang-II was decreased in pregnant saline infused rats as compared with non-pregnant rats. These changes were not observed during preeclampsia.

Conclusion

Pregnancy induced adaptations in endothelial function, which were not observed in the rat model for preeclampsia. This role of lack of pregnancy induced endothelial adaptation in the pathophysiology of experimental preeclampsia needs further investigation.

INTRODUCTION

Preeclampsia is a pregnancy specific syndrome, clinically characterized by the presence of hypertension, associated with proteinuria in the second half of pregnancy [1]. Preeclampsia complicates about 5% of pregnancies and is a leading cause of maternal and perinatal mortality [1]. The etiology of preeclampsia remains unknown, but appears to be related to the presence of the placenta [2]. In preeclamptic patients physiological remodelling of the uterine spiral arteries is diminished, resulting in decreased placental perfusion [3]. Several mechanisms have been implicated in the pathophysiology of preeclampsia, including activation of inflammatory cells [4], endothelial cell activation and vascular dysfunction [5], as well as changes in the renin-angiotensin-aldosterone system (RAAS) [6].

During normal pregnancy, vascular function changes dramatically; increased endothelium dependent vascular relaxation as well as increased flow mediated dilation can be observed [7]. Together this may result in a decrease in blood pressure (mainly in the second trimester) and a decrease in peripheral vascular resistance [8]. By production of vasoactive factors, endothelial cells are important mediators of vascular tone [9]. Changes in the production of these vasoactive factors may therefore account for the pregnancy-related changes in vascular relaxation. Indeed, the production of endothelial prostacyclin, nitric oxide (NO) as well as the unidentified endothelium-derived hyperpolarizing factor (EDHF) has been shown to be increased during pregnancy [10–13]. In contrast to normal pregnancy, vascular relaxation is reduced in preeclampsia [8]. The endothelial cell dysfunction in preeclampsia appears to be associated with an impaired regulation and secretion of vasodilating factors, such as NO, prostacyclin production or EDHF [12,14,15].

In addition, the RAAS may also be involved in the changes in vascular dysfunction in preeclampsia. While normal pregnancy is associated with a decreased sensitivity to the vasoconstrictor angiotensin II (Ang-II) [16], preeclampsia is associated with an increased response to Ang-II as compared to normal pregnancy [17]. During preeclampsia, the increased Ang-II sensitivity may even develop before the clinical manifestation of the disease [18,19]. Ang-II exerts its effects via two receptors. Binding of Ang-II to the Ang-II Type I receptor (AT1-R) causes contraction [17]. The other Ang-II receptor is the Type II receptor (AT2-R). The function of this receptor is less well understood. There is however, increasing evidence that the AT2-R may exert an inhibitory influence on AT1-R mediated stimulation [20]. It is largely unknown if and how these receptors are involved in the changes in Ang-II sensitivity during normal pregnancy and preeclampsia. However, it seems likely that the AT1-R is involved in the pathophysiology of preeclampsia, since in both rat and mice it has been demonstrated that treatment with AT1-R blockers inhibited the development of clinical signs in models of preeclampsia [21,22]. Unfortunately, treatment with AT1-R blockers is contraindicated during pregnancy [23].

In the present study, we evaluated endothelial function during pregnancy and experimental preeclampsia in the rat, by studying the role of the vasoactive factors in endothelial function as well as the role of the AT1-R and AT2-R in the Ang-II sensitivity. We used the well-established model for preeclampsia, i.e. the low-dose lipopolysaccharide (LPS) infused pregnant rat [24]. This model is

characterized by hypertension and proteinuria and has been used as a model for preeclampsia for many years and was used in many studies [21,25–27], including a recent study by Wang et al. [28].

MATERIALS AND METHODS

Animals

Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Ethical Experiments of the University of Groningen (application number: DEC-5516A).

Female Wistar outbred rats (Harlan Inc, Horst, the Netherlands) were kept in a 12 hour light-dark cycle and constant room temperature, with food and water available ad lib in the home cages. Until selection for experiments vaginal smears were taken daily. Rats were rendered pregnant by housing them on pro-oestrus with fertile males for one night. Day 0 of pregnancy was documented by the presence of spermatozoa in the vaginal smear. In cyclic and pregnant rats, the latter ones on day 0 of pregnancy, a cannula was inserted into the right jugular vein under isoflurane/oxygen anesthesia according to standard methods [29]. The jugular vein cannula allows stress free infusion. On day 14 of pregnancy or 14 days after cannula placement, infusion of either saline or LPS took place. Day 14 of pregnancy was chosen since in the rat trophoblast invasion into the mesometrial triangle, i.e. the equivalent of the placental bed, and the spiral arteries starts around this day of pregnancy.

The low-dose LPS treated pregnant rat is an established model of preeclampsia, characterized by hypertension, proteinuria, disseminated intravascular coagulation, generalized activation of the inflammatory response and endothelial cell activation [24,28,30]. LPS is infused at day 14 during 1 hour. The final concentration of LPS immediately after the infusion is found to be very low or even undetectable in some rats and from fifteen minutes onward undetectable in all rats (unpublished results). The development of the preeclamptic-like syndrome in this model is considered to result from a systemic inflammatory response induced by LPS [25,26,30]. In addition to studies focussing on the pathophysiology of preeclampsia, studies concerning therapeutic options for preeclampsia have also been performed in this model [27,28,31]. The rats were randomly divided into four groups as follows: non-pregnant (NP) saline infused (2 ml in 1 hour); pregnant (P) saline infused (2 ml in 1 hour); P LPS (E-Coli, 0.55: B5, Whittaker MA Bioproducts, Walkerville, Md.) infused (1µg/kg bw in 2 ml saline in 1 hour); NP LPS infused (1µg/kg bw in 2 ml saline in 1 hour). P-saline infused rats served as healthy pregnant controls whereas the LPS infused pregnant rats served as the preeclampsia group [24]. Six days after infusion (on day 20 of pregnancy), the animals were anesthetized with isoflurane/oxygen and decapitated. The aorta was isolated and placed in cold oxygenated Krebs solution. The number of pups was counted and their length as well as maternal weight measured.

Drugs and chemicals

Krebs buffer (pH 7.4) was freshly made before the start of each experiment and contained in mmol/L: 120 NaCl, 5.9 potassium chloride (KCl), 25.2 $NaHCO_3$, 1.2 NaH_2PO_4, 10.4 glucose, 1.21 $MgCl_2 \cdot 6H_2O$, and 2.52 $CaCl_2$. All Krebs ingredients were purchased from E. Merck (Darmstadt, Germany). The stock solutions for phenylephrine (Sigma, St. Louis, MO, USA), acetylcholine (Sigma, St. Louis, MO, USA), Ang-II (Bachem AG, Bubendorf, Switzerland), PD-123319 (Park-Davis), Losartan (Merck Research laboratories, Rahway, USA), and N^G-nitro-L-arginine methyl ester (L-NMMA; Calbiochem Brand of EMD Biosciences, Inc., La Jolla) were prepared in saline (0.9%NaCl in distilled water). Indomethacin (Sigma) was dissolved in $NaHCO_3$.

Aortic-ring contraction studies

The endothelium-dependent relaxation and sensitivity to Ang-II in aortic tissue was studied by standard isotonic contraction experiments with thoracic aorta rings of the rat as previously described [32,33]. Aortic rings (2mm) from the rats were kept in Krebs solution (at 37°C) and gassed with 95% CO_2 and 5% O_2. Prior to priming the aortic rings were equilibrated for 30 minutes and subsequently checked for viability by evoking a contraction with KCl (60mM) for 10 minutes. Excess aortic tissue was snap frozen and kept in -80°C.

Endothelium-dependent relaxation

Eight aortic rings of each rat were used to study the endothelium-dependent relaxation. The rings were studied in duplo in the continuous presence of either vehicle, NO synthase inhibitor L-NMMA (10^{-4}M), cyclooxygenase (COX) inhibitor indomethacin (10^{-5}M) or with L-NMMA plus indomethacin, to study the resultant role of EDHF. After 20 minutes of pre-incubation, aortic rings were pre-contracted with 10^{-6}M phenylephrine. Then, increasing concentrations of acetylcholine (10^{-8}M-10^{-4}M) were added to the medium to investigate endothelium-dependent dilation after stabilization. Subsequently, the NO donor sodium nitroprusside (SNP; 10^{-5}M) was added as a control for endothelium-independent relaxation. The mean acetylcholine-mediated relaxation of the two rings in each condition was calculated as a percentage of the phenylephrine mediated pre-contraction.

Response of the aortic rings to Ang-II

Functional response of the AT1-R to Ang-II

To determine the Ang-II induced contractile response via the AT1-R, two aortic rings of each rat were pre-incubated for 20 minutes with 10^{-6}M PD-123319, an AT2-R antagonist [34] and the selective NO synthase inhibitor L-NMMA (10^{-4}M) to prevent any confounding effects by the basal release of NO [35]. Both compounds were present during the entire experiment. Then, a cumulative Ang-II concentration-response curve (10^{-10}M-10^{-6}M) was obtained according to standard methods [36,37]. A subsequent amount of Ang-II was added after renewed stabilization. Following the response curve, a reference contraction response was evoked by stimulation with 10^{-5}M phenylephrine and after stabilization of the phenylephrine response KCl (60mM) was added, to produce maximal contraction. The mean Ang-II-mediated contraction of the duplo rings in each condition was calculated as a

percentage of the maximal KCl induced response.

Functional response of the AT2-R to Ang-II

To determine the functional response of the AT2-R to Ang-II, two aortic rings of each rat were pre-incubated for 20 minutes with 10^{-5}M losartan, an AT1-R antagonist [38]. This antagonist was present during the entire experiment. Following the pre-incubation period, the aortic rings were pre-contracted with 10^{-6}M phenylephrine. After pre-contraction reached a stable contraction, cumulative Ang-II concentration-response curve (10^{-10}M-10^{-6}M) was obtained according to standard methods [36,37]. Thereafter, the NO donor SNP (10^{-5}M) was added to the aortic rings, as a control for maximal endothelium-independent relaxation. For each rat, and for each dose of Ang-II, the mean Ang-II mediated vasodilatory response for the two rings was calculated as a percentage of the maximal pre-contraction with phenylephrine.

Gene-expression analysis

Total aortic RNA was isolated with TriReagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Total RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA synthesis was performed as described before [39]. Real time quantitative PCR was performed using an Applied Biosystems 7900 FAST sequence detector (Foster City, California, USA) and Applied Biosystems reagents according to the manufacturer's instructions. Expression levels were normalized to those of 18S ribosomal RNA which was analyzed in separate runs. Primers and probes for the AT1-R and AT2-R were obtained from Applied Biosystems (TaqMan Gene Expression Assays, AT1-R: Rn00578456_m1 and AT2-R: Rn00560677_m1). Primers and probes for iNOS, eNOS, COX1, COX2 and 18S ribosomal RNA were obtained from Invitrogen (Breda, Invitrogen). The sequences were (sense primer, antisense primer, and probe, respectively; all from 5' to 3'): 18S (M11188), CGGCTACCACATCCAAGGA, CCAATTACAGGGCCTCGAAA, CGCGCAAATTACCCACTCCCGA. Cox1 (XM_579388.1), CCCAGAGTCATGAGTCGAAGG, AACAACAGGGATTGACTGGTGA, TTTCCCCTGCTGCTGCTCCTGC. Cox2 (NM_017232.2), TTGTTGAGTCATTCACCAGACAGAT, GCCTTTGCCACTGCTTGTACA, CCCCAGCAACCCGGCCAGC. Enos (NM_021838.2), AGGAAGTAGCCAATGCAGTGAA, AGCCATACAGGATAGTCGCCTT, CGCTTCGCCATCACCGTGCC. Inos (NM_012611), CTATCTCCATTCTACTACTACCAGATCGA, CCTGGGCCTCAGCTTCTCAT, CCCTGGAAGACCCACATCTGGCAG.

The expression levels of AT1-R, AT2-R, iNOS, eNOS, COX1 and COX2 were normalized to those of 18S ribosomal RNA.

Statistics

Statistical analysis was performed using SPSS for Windows (Version 16.0), the EC_{50} and E_{max} were calculated using GraphPad Prism 5, on a standard computer. The independent sample T-test was used to analyze differences between the number and length of the rat pups. Two-way analysis of variance (Two-way ANOVA) was used to analyze differences in bodyweight of the rats. We show the dose response curves of all four groups after vehicle incubation. Whether there was a significant difference between the four groups of rats was tested using General Linear Model for repeated measures. To calculate whether NO, PG or EDHF played a significant role in the acetylcholine mediated relaxation the E_{max} of the different curves was analyzed and compared with the E_{max} of the vehicle curve (i.e. total relaxation) using Student t-test. To test differences in contribution of a certain factor between the four groups of rats, Two-way ANOVA was used. If the Two-way ANOVA detected significant differences, we tested whether P-saline infused rats differed from NP-saline infused rats and whether LPS infused rats differed from saline infused rats in both pregnant and non-pregnant groups, using independent student T-test with Bonferroni corrections. For the Ang-II response curves the E_{max} and EC_{50} were calculated to represent individual responses. Differences in the E_{max} and EC_{50} of the Ang-II response curves between the four groups were analyzed using Two-way ANOVA to detect an effect of pregnancy or LPS infusion. If the Two-way ANOVA detected significant differences, we tested whether P-saline infused rats differed from NP-saline infused rats and whether LPS infused rats differed from saline infused rats in both pregnant and non-pregnant groups, using independent student T-tests with Bonferroni corrections. To test whether correlations existed between Ang-II mediated relaxation through the AT2-R and the vasoactive role of NO, PG or EDHF in acetylcholine-mediated relaxation Pearson Correlation test were performed. PCR data are presented as relative gene expression to 18S ribosomalRNA and analyzed using Two-way ANOVA with log transformed data. We tested whether P-saline infused rats differed from NP-saline infused rats and whether LPS infused rats differed from saline infused rats in both pregnant and non-pregnant rats, using independent student T-test with Bonferroni corrections. In all cases, differences were considered significant if $p \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

The body weight was significantly increased in pregnant rats compared to non-pregnant rats ($p<0.001$). No significant effect of treatment (saline or LPS infusion) was found ($p=0.093$). The number of pups was not significantly different between the two pregnant groups ($p=0.329$). However, the length of the pups in the P-LPS infused rats was significantly lower compared to the P-saline rats ($p=0.01$). The number of resorptions did not differ between these groups, one animal of each group showed one resorption. Table 1 presents these rat characteristics.

Table 1. Rat characteristics

Group (n)	NP-saline (8)	P-saline (8)	P-LPS (9)	NP-LPS (8)
Maternal weight (g)	242.12 (7.4)	325.50* (6.9)	344.22* (8.5)	249.75 (6.9)
Number of pups	-	12.0 (1.0)	13.44 (1.0)	-
Length pups (mm)	-	32.36† (0.22)	31.57 (0.21)	-

P= pregnant; NP=non-pregnant; LPS= Lipopolysaccharide.

*Data are presented as mean ±SEM. n=number of rats in group. *: p<0.05 vs NP; †: p<0.05 vs P-LPS.*

Endothelium-dependent relaxation in aortic rings

To study endothelial function in pregnancy and experimental preeclampsia in the rat, we used aortic rings, precontracted with phenylephrine and dilated with acetylcholine in the presence of vehicle, L-NMMA, to identify the role of NO, indomethacin, to identify the role of PG or L-NMMA and indomethacin, to identify the role of other factors then NO or PG, mainly EDHF. There was no effect of pregnancy or treatment (LPS or saline infusion), nor of the various inhibitors on the contraction with KCl prior to the start of the experiment (results not shown). Also, precontraction with phenylephrine following the incubation with the various inhibitors did not differ between the four groups, apart from the increased precontraction in P-saline rats vs the other three groups after indomethacin incubation (results not shown). Moreover, SNP evoked endothelial independent relaxation was decreased in P-saline rats vs NP-saline rats following incubation with all inhibitors (results not shown).

After vehicle incubation, all routes for relaxation are available and by adding acetylcholine in a cumulative fashion after precontraction with phenylephrine, relaxation appeared equal in all four groups (Figure 1). No significant difference in total acetylcholine mediated relaxation was seen between the four groups. Moreover, no significant differences in -logEC_{50} or E_{max} were found between the four groups (Table 2; acetylcholine relaxation and Figure 2B).

The contribution of the different factors to the acetylcholine mediated relaxation is presented in Figure 2. Figure 2A shows the curves after vehicle, L-NMMA, indomethacin, and L-NMMA plus indomethacin incubation in NP-saline infused rats as an example. This figure shows that when aortic rings were relaxated in the presence of L-NMMA, which inhibits NO production, we observed a significantly decreased relaxation. This curve represents the resultant relaxation in the absence of NO. The curve after incubation with indomethacin, which inhibits the production of PG, represents the resultant relaxation in the absence of PG. In the presence of indomethacin, we observed a significant increase in relaxation as compared with the curve after vehicle incubation. This increase in relaxation following indomethacin indicates that the aorta produced mainly contractile PG (Figure 2A). When aortic rings were incubated with both L-NMMA and indomethacin, production of both NO and PG are inhibited, the curve therefore represents the resultant relaxation, which is due to other factors, i.e. EDHF. After incubation of aortic rings with L-NMMA and indomethacin, relaxation

was significantly decreased as compared with the relaxation after vehicle incubation (Figure 2A).

Table 2. -logEC$_{50}$ dose response curves

Group	NP-saline	P-saline	P-LPS	NP-LPS
Acetylcholine relaxation (total relaxation)	6.82 ± 0.21	6.87 ±0.28	6.86 ± 0.06	6.63 ± 0.56
Acetylcholine relaxation in presence of L-NMMA	6.74 ± 0.29	6.58 ± 0.10	6.23 ± 0.52	6.79 ± 0.28
Acetylcholine relaxation in presence of Indomethacin	7.25 ± 0.13	6.98 ± 0.12	7.00 ± 0.16	7.24 ± 0.08
Acetylcholine relaxation in presence of L-NMMA + indomethacin	6.65 ± 0.14	6.69 ± 0.25	6.89 ± 0.17	6.45 ± 0.17
Ang-II contraction	7.92 ± 0.10*	7.56 ± 0.38	8.12 ± 0.11*	7.75 ± 0.10
Ang-II relaxation	8.33± 0.52	10.21 ± 3.74	9.45 ± 0.80	9.48 ± 0.40

*P= pregnant; NP=non-pregnant; LPS= Lipopolysaccharide. Two-way ANOVA showed no effect of pregnancy or treatment (saline or LPS infusion) for acetylcholine mediated relaxation, for L-NMMA, indomethacin and L-NMMA plus indomethacin, and for Ang-II induced relaxation. Two-way ANOVA did shown an interaction between pregnancy and treatment for Ang-II mediated contraction (p=0.0122). Therefore, Student T-tests were performed with Bonferroni corrections. Data are presented as mean ± SEM. *: p<0.001 vs P-saline.*

Figure 2B shows the E_{max} of the acetylcholine induced relaxation after incubation with vehicle, L-NMMA, indomethacin or L-NMMA and indomethacin of aortic rings of the four groups of rats. Since the E_{max} for NO and PG was calculated after incubation with their blockers (L-NMMA and indomethacin respectively), a higher E_{max} represents a lower NO or PG production. Since the curve after L-NMMA plus indomethacin incubation represents the relaxation due to EDHF, a higher E_{max} for this curve, represents a higher EDHF production.

The total relaxation, i.e. relaxation after vehicle incubation, did not differ between the groups (Figure 2B, first graph). Also no difference in -logEC$_{50}$ for total relaxation was observed between the groups (Table 2). After incubation of the aortic rings with L-NMMA, when NO production is inhibited, the E_{max} is very low and significantly decreased from the E_{max} after vehicle incubation in all groups, with no differences between the groups. This indicates that the resultant relaxation in the absence of NO is very small. NO thus play a large role in the relaxation of the aorta in all groups (Figure 2B, second graph). Also no difference in -logEC$_{50}$ after L-NMMA incubation was observed between the groups (Table 2). After incubation with indomethacin, when PG production is inhibited, the E_{max} is significantly increased compared to the E_{max} of the vehicle curve, in NP-saline and LPS infused rats and in P-LPS infused rats (Figure 2B, third graph and Table 2).

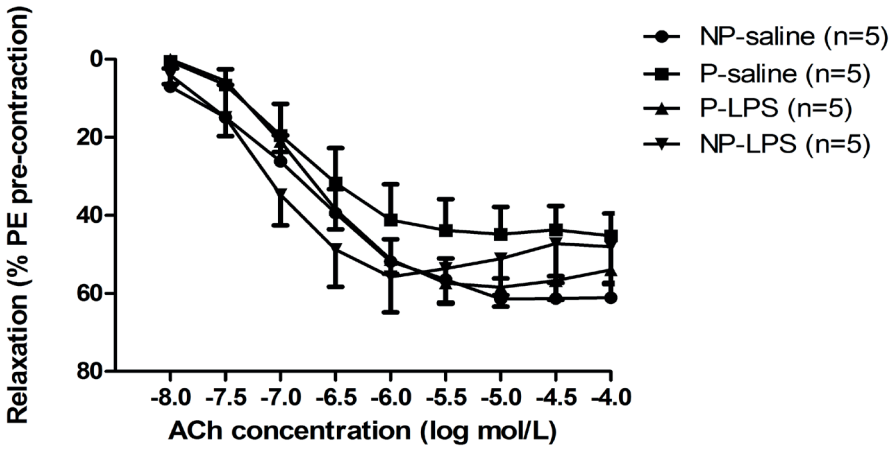

Figure 1. Endothelium dependent relaxation.

The mean ± SEM acetylcholine-mediated endothelium dependent relaxation in the thoracic aorta of non-pregnant saline (NP-saline; circle), pregnant saline (P-saline; square), pregnant-LPS (P-LPS; triangle upward), and non-pregnant-LPS (NP-LPS; triangle downward) infused rats after incubation with vehicle.

The percentage relaxation was calculated as percentage of the pre-contraction with phenylephrine (PE). Analyzing the data with General Linear Model of repeated measures showed no significant differences between the curves in the four groups.

Thus in the absence of PG, the aorta's showed an increased relaxation, indicating that in these aorta's mainly contractile PG are produce. In P-saline infused rats, however, the E_{max} after indomethacin incubation was not different from the E_{max} following vehicle incubation, suggesting that PG are not produced by aorta of these pregnant rats. The E_{max} from P-saline infused rats is significantly lower than the E_{max} of the other three groups. However, no difference in -logEC_{50} after indomethacin incubation was observed between the groups (Table 2).

The last graph of Figure 2B shows the E_{max} after incubation with L-NMMA and indomethacin, i.e. when both NO and PG are inhibited. This E_{max} represents the relaxation due to other factors than NO and PG, i.e. EDHF. The resultant relaxation due to EDHF is low, indication a minor role for EDHF in the contraction of the aorta after acetylcholine and significantly decreased from the E_{max} after vehicle incubation in all groups except in the NP-LPS infused rats. However, the E_{max} after L-NMMA and indomethacin incubation was significantly increased in P-LPS infused rats as compared to the E_{max} of the P-saline infused rats, suggesting a more important role for EDHF in acetylcholine-induced aortic relaxation in P-LPS as compared to P-saline infused rats. However, no difference in -logEC_{50} after L-NMMA and indomethacin incubation was observed between the groups (Table 2).

These data thus show that pregnancy induced a shift in components inducing relaxation compared to NP rats, which is annihilated in preeclampsia.

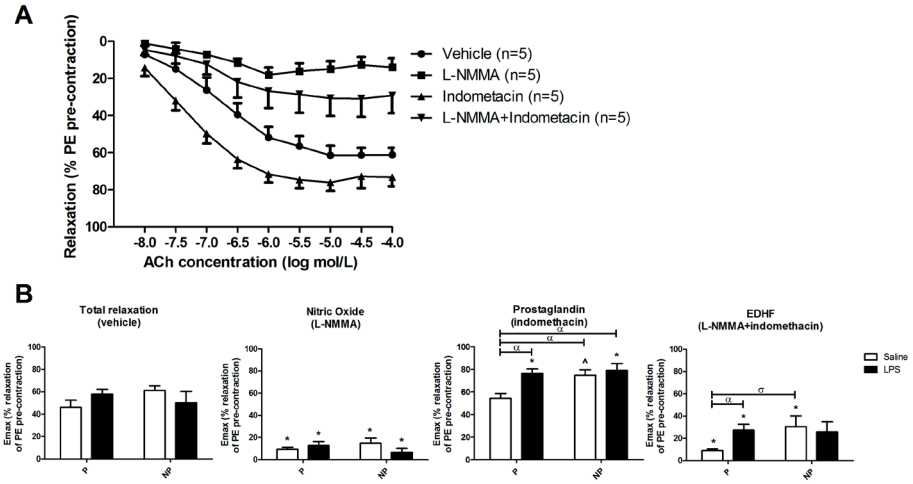

Figure 2. Endothelium dependent relaxation – contribution of the different factors.

(A) The mean ± SEM acetylcholine-mediated endothelium dependent relaxation in the thoracic aorta of the non-pregnant saline infused rats after vehicle incubation (total relaxation; circle), after L-NMMA incubation (nitric oxide; square), after indomethacin incubation (prostaglandin; pyramid upward), and after L-NMMA and indomethacin incubation (EDHF; pyramid downward). The percentage relaxation was calculated as percentage of the pre-contraction with phenylephrine (PE).

*(B) The E_{max} of the endothelium dependent relaxation under the different conditions in the thoracic aorta from pregnant rats (P; left set of bars) and non-pregnant rats (NP; right set of bars) infused with saline (white bars) or lipopolysaccharide (LPS; black bars). Data are presented as mean ± SEM. *: $p<0.05$ vs E_{max} of the total relaxation within the same group of rats (Student T-test). α: $p<0.05$ vs P-saline; σ: $p<0.1$ vs P-saline (Two-way ANOVA). A trend towards a significant interaction between pregnancy and treatment (saline or LPS) was found for prostaglandin ($p=0.09$).*

Endothelium mRNA expression

Four types of enzymes related to the production of NO and PG were analyzed for expression of their mRNA level in thoracic aortas. mRNA expression of endothelial and inducible nitric oxide synthase (eNOS and iNOS respectively) was not different between the four groups. Also, mRNA expression of COX-1 and COX-2 was not different between the four groups. Results are shown in Figure 3.

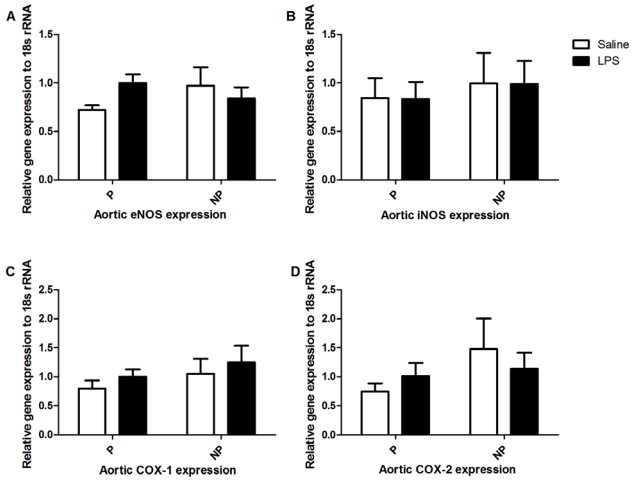

Figure 3. mRNA expression of eNOS, iNOS, COX-1 and COX-2 in aortic tissue.

The mRNA expression of eNOS (A), iNOS (B), COX-1 (C) and COX-2 (D) in aortic tissue from pregnant-saline (left set of bars) and non-pregnant (right set of bars) infused with saline (open bars) or LPS (black bars). Two-way ANOVA showed no effect of pregnancy or treatment (saline or LPS infusion).

Response of aortic rings to Ang-II

Ang-II mediated contraction

Figure 4 represents the cumulative Ang-II contraction curves (E_{max} shown in inset). In the presence of the AT2-R blocker PD-123319, when Ang-II can only bind to the AT1-R, contraction was observed in all groups. However, Ang-II mediated contraction was significantly blunted in P-saline infused rats (significantly decreased E_{max}) compared to NP-saline infused rats (p=0.007). Moreover, after LPS infusion in P-rats a significant increase in Ang-II mediated contraction was seen as compared to the P-saline infused rats (p=0.017). There was, however, no effect of LPS treatment compared to saline infusion pertaining to the response of the AT1-R to Ang-II in NP-rats (p=0.713). Table 2 shows the -logEC_{50} of the Ang-II mediated contraction dose response curves. The -logEC_{50} was significantly increased in P-LPS infused rats as compared P-saline infused rats (p<0.001). Moreover, the -logEC_{50} was significantly increased in NP-saline infused rats as compared to P-saline infused rats (p<0.01). Thus, the pregnancy related decrease in Ang-II mediated contraction is absent in preeclampsia.

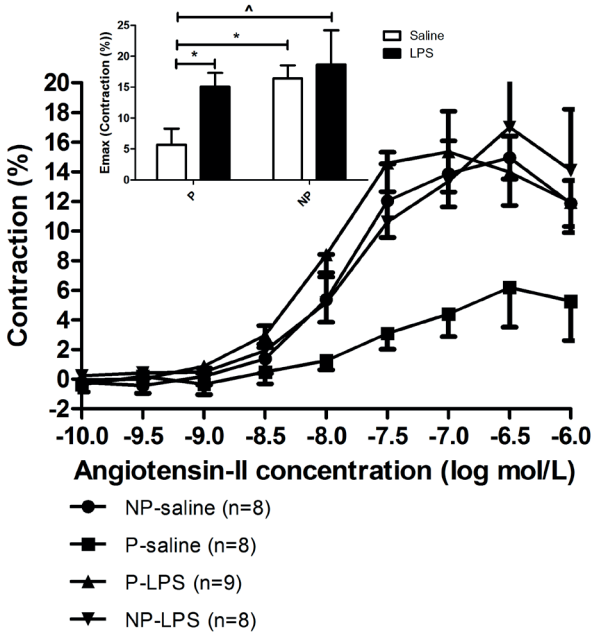

Figure 4. Cumulative Ang-II contraction response curves.

The mean ± SEM cumulative Ang-II contraction curves in the thoracic aorta from the female rat in the non-pregnant-saline (NP-saline; circle; n=8), the pregnant-saline (P-saline; square; n=9), the pregnant-LPS (P-LPS; pyramid upward; n=9), and the non-pregnant-LPS (NP-LPS; pyramid downward; n=8) group. Percentages are calculated as percentage of the maximum contraction reached after adding 10^{-5}M phenylephrine and 60mM KCl, at the end of the concentration response curves. Inset: Mean ± SEM E_{max} of the cumulative contraction curves. Two-way ANOVA showed a significant effect of pregnancy(p=0.05) and a trend for treatment (p=0.1), with no interaction effect between pregnancy and treatment (p=0.315). The effect of pregnancy and treatment was further analyzed with Student T-test using Bonferroni corrections.: p<0.05; ∧: p<0.1.*

Ang-II mediated relaxation through the AT2-R

Figure 5 represents the cumulative Ang-II dilation response curves (E_{max} shown in inset). In the presence of the AT1-R blocker losartan, when Ang-II can only bind to the AT2-R, relaxation was observed in all but the P-saline infused rats. The response upon Ang-II (i.e. the E_{max}) was significantly blunted in P-saline compared to NP-saline infused rats (p<0.001). Infusion of LPS in pregnant rats increased the Ang-II mediated dilation compared to P-saline infused rats (p=0.05). There was no effect of LPS on the Ang-II sensitivity in non-pregnant rats (p=0.06). No significant differences were found between the four groups in the -logEC_{50} of the Ang-II mediated relaxation through the AT2-R dose response curves (Table 2).

Thus, preeclampsia seems to reverse the decrease in Ang-II mediated relaxation through the AT2-R seen in healthy pregnancy compared to the non-pregnant situation.

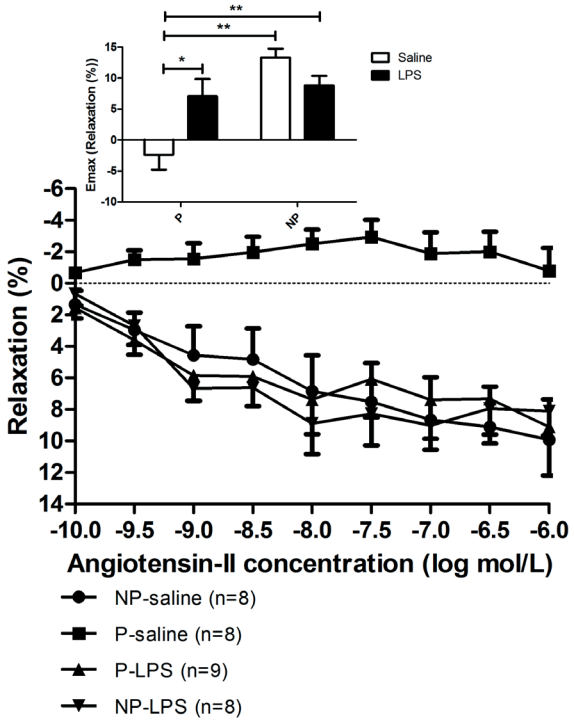

Figure 5. Cumulative Ang-II dilation response curves.

*The mean ± SEM cumulative Ang-II dilation curves of the in the thoracic aorta of non-pregnant saline (NP-saline; circle), pregnant saline (P-saline; square), pregnant-LPS (P-LPS; triangle upward), and non-pregnant-LPS (NP-LPS; triangle downward) infused rats. Percentages are calculated as percentage contraction upon Ang-II of the maximum contraction reached after adding 10^{-6}M phenylephrine. Inset: Mean ± SEM E_{max} of the cumulative Ang-II dilation curves. Two-way ANOVA showed a significant effect of pregnancy (p=0.001), with an interaction effect between pregnancy and treatment (p=0.006). The effect of pregnancy and treatment was further analyzed with Student T-test using Bonferroni corrections. *: p<0.05; **: p<0.01.*

Ang-II receptor expression

There was no effect of pregnancy or treatment (LPS or saline infusion) on AT1-R or AT2-R mRNA expression in aortic tissue. However, the ratio between the AT1-R and the AT2-R mRNA in aortic tissue showed a significant effect of treatment. The LPS-infused rats showed a significant higher ratio (Figure 6) compared to the saline-infused rats, regardless of pregnancy (p=0.03).

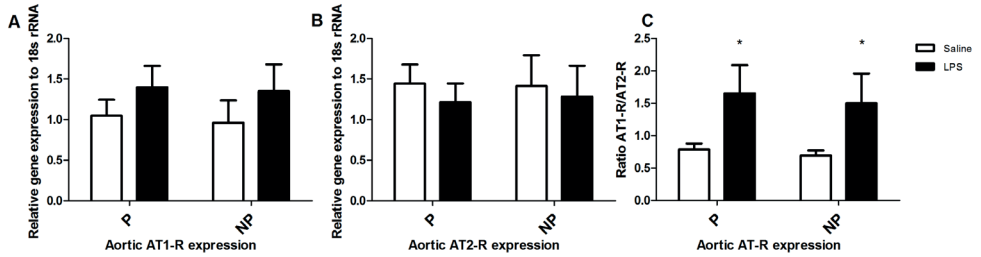

Figure 6. mRNA expression of AT1-R and AT2-R in aortic tissue.

The mRNA expression of the AT1-R (A) and the AT2-R (B) in aortic tissue from pregnant-saline (left set of bars) and non-pregnant (right set of bars) infused with saline (open bars) or LPS (black bars). Values for AT1-R and AT2-R mRNA were normalized to those of 18S ribosomal RNA. Two-way ANOVA was used to analyze the data. No significant differences were found between either LP and NP, saline infusion and LPS infusion or the interaction effect between pregnancy and treatment, in aortic tissue.

(C.) The ratio of the AT1-R and AT2-R mRNA expression in aortic tissue from pregnant-saline (left set of bars) and non-pregnant (right set of bars) infused with saline (open bars) or LPS (black bars). Values for AT1-R and AT2-R mRNA were normalized to those of 18S ribosomal RNA. The ratio was calculated by dividing AT2-R to AT1-R. Using two-way ANOVA a significant effect of treatment (p=0.03) was found in aortic tissue, independent of pregnancy.

**:p<0.05, LPS versus saline.*

Correlation endothelium-dependent relaxation and response to Ang-II

The Ang-II relaxation mediated through the AT2-R significantly correlated with the contribution of EDHF (r=0.535, p=0.018) (Figure 7). No significant correlation was found between Ang-II mediated relaxation through the AT2-R and the contribution of NO (r=0.020, p=0.934; data not shown) and between Ang-II mediated relaxation through the AT2-R and the contribution of PG (r=0.411, p=0.081; data not shown).

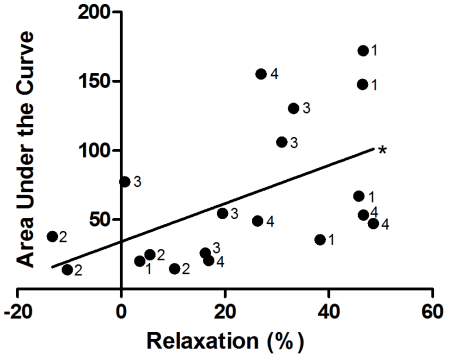

Figure 7. Correlation contribution of EDHF and Ang-II mediated relaxation through the AT2-R.

*The correlation between contribution of EDHF (AUC) and the Ang-II mediated relaxation through the AT2-R (relaxation (%)) in all groups. 1=non-pregnant-saline group; 2=pregnant-saline group; 3=pregnant-LPS group; 4=non-pregnant-LPS group. *:Pearson Correlation test, r=0.535, p<0.05.*

DISCUSSION

In this study, we investigated endothelial function by studying the endothelium-dependent relaxation and the role of the AT1-R and AT2-R in the Ang-II sensitivity of healthy control pregnant and preeclamptic rats [24] using aortic rings in an organ bath setup for isotonic displacement [40]. The model used for experimental preeclampsia, i.e. the low dose LPS-infused pregnant rat is an established model for preeclampsia, which has been used for many years [25,26]. It is not only characterized by hypertension and proteinuria in the last week of pregnancy [24,28], but also with endothelial cell activation and generalized inflammation [25]. Unfortunately, due to the experimental setup we were not able to measure blood pressure in this study. However, as stated above, the model used is an established model for studying preeclampsia and all studies performed, including recent studies in our lab, showed increase in blood pressure in this preeclampsia rat model.

We found that during pregnancy, there appeared to be a decreased role for contractile PG and a decreased role for EDHF in acetylcholine-induced relaxation of the aorta as compared to the non-pregnant state. We also found that the Ang-II sensitivity was blunted during pregnancy: we demonstrated a decreased response upon binding of Ang-II to the AT1-R (vasoconstriction) as well as upon binding to the AT2-R (vasorelaxing) in P-saline infused rats compared with NP-saline infused rats. Interestingly, similar to human preeclampsia, the pregnancy-induced changes in endothelium-dependent relaxation as well as in the decrease in contraction and relaxation response upon Ang-II were not observed in experimental preeclampsia in the present study. In fact, the endothelium-dependent relaxation and the responses upon Ang-II in experimental preeclamptic rats were comparable to those in the non-pregnant rats.

Incubation of aortic rings with vehicle showed no differences in relaxation between the four groups, suggesting that the total vascular vasoactive capacity of the aorta is not altered during normal pregnancy or during preeclampsia in the rat. This does not corroborate with some other studies [41–43], but it is in line with the study of Ballejo et al., who also showed that endothelium dependent relaxation in response to acetylcholine is not altered in late pregnancy [44]. It also agrees with various other studies showing that acetylcholine induced relaxation does not differ in non-pregnant vs late pregnant rats [45–47]. Differences in results may be due to strain differences [48] or to differences in timing of termination of pregnant or non-pregnant animals, differences in vascular bed used or differences in experimental methods. However, SNP treatment at the end of the experiments, showed a significantly attenuated relaxation in pregnant rats compared to non-pregnant rats (results not shown). This may indicate that during pregnancy, relaxation in the aorta is more dependent on endothelium-derived factors than in the non-pregnant state. Other models for preeclampsia, TNF infusion [49] or IL-6 infusion [50] in pregnant Sprague-Dawley rats did show decreased total vascular reactivity in the aorta as compared with control rats. These are, however, other models, in another rat strain, which may explain the differences.

In our study, also the role of NO in the vasodilatory capacity of the aorta did not differ between pregnant and non-pregnant rats. Although this suggestion is not in line with some previous studies [51,52], it agrees with others [44,46]. This lack of differences in role of NO between the groups in

our study is in accordance with the lack of differences in aortic iNOS or eNOS expression between the groups. Various studies have suggested a role for NO in the relaxation in pregnancy and the contraction in preeclampsia. Indeed, some studies showed that endogenous production of NO and eNOS mRNA are increased in pregnant rats [38,53]. Also *in-vivo* treatment of rats with L-NMMA induced a higher increase in blood pressure in pregnant as compared to non-pregnant rats suggesting an increased role for NO in vascular vasoactive responses in pregnant versus non-pregnant rats [54]. It may be suggested that our lack of difference in the role of NO in the aorta may be due to the fact that endothelial NO production during pregnancy may be enhanced spontaneously or in response to vasoconstricting agents, but not in response to vasorelaxing agents [44]. As suggested above, differences in responses may also be due to strain differences, since vascular responses to pregnancy are generally lower in Wistar rats as compared to Sprague-Dawley rats [48]. Also differences in vascular beds used may account for differences between studies, since the aorta, which is a conduit vessel, and largely depends on NO, may respond differently than a mesenteric vessel, which is a resistance vessel and depends to a much lesser extend on NO [55]. However, methodological differences or differences in timing of pregnancy may also play a role.

In contrast to NO, the involvement of vasoactive PG in acetylcholine-induced relaxation responses appeared to change during pregnancy in the present study. Changes in PG in pregnancy have also been found by Bobadilla et al. [56], but not by others studies (including another study of Bobadilla et al. [52,57]. As described above, differences might be due to difference in strain used and methodological differences since Aloamaka et al. studied responses upon vasocontractile agents. In NP rats, inhibition of PG with indomethacin enhanced acetylcholine-induced relaxation, indicating the involvement of contractile PG in rats. However, this effect was absent in P-saline infused rats, suggesting that pregnancy was associated with a larger role of vasorelaxing PG, such as prostacyclin, in endothelium dependent relaxation. Alternatively, a decrease in contractile PG or receptor down regulation of the prostaglandin route during pregnancy may also be suggested. This observation is strengthened by the observation that precontraction with phenylephrine after incubation with indomethacin is enhanced in P-saline infused rats as compared with the other 3 groups of rats. These data are in line with the suggestion that vasodilatory PG may oppose the action of vasoconstrictors in pregnancy [58]. As incubation with indomethacin caused an increase in relaxation in P-LPS infused rats, this putative role of prostacyclin during pregnancy is blunted in experimental preeclampsia. With these results, our findings seem to be in line with results in human preeclampsia [59–63], which showed decreased prostacyclin production in preeclampsia versus normal pregnancy [64], as well as with other models of preeclampsia [65,66]. The altered involvement of vasoactive PG in acetylcholine-induced relaxation responses found in our study, appeared independent of regulation of COXs expression, since we found no differences in mRNA expression of COX-1 or COX-2. However, we take into account that mRNA expression is not a surrogate for protein expression or post-translational effects in target cells.

The role of EDHF in endothelium-dependent relaxation was studied using concomitant incubation of the aortic rings with L-NMMA and indomethacin. This results in inhibition of NO and PG, therefore the resultant relaxation response is due to EDHF, or other unknown factors,

such as hydrogen sulfide [67] by means of exclusion. EDHF is an endothelium-derived relaxing factor that causes vasorelaxation in association with vascular smooth muscle hyperpolarization [68]. The chemical identity of EDHF is uncertain [13]. In our study in aortic rings, EDHF or these other factors significantly contributed to acetylcholine-induced relaxation in all groups, but was of significantly of less importance in P-saline infused rats. Other studies comparing the role of EDHF during pregnancy found an increased role for EDHF in pregnancy [52,69]. However, these studies were performed in mesenteric arteries. Results may be different in humans, since EDHF was found to play a significant role in myometrial and subcutaneous arteries of pregnancy compared to preeclampsia [15,70]. This inconsistency in our rat model may also be explained by the fact that different arteries were used, since it is well known that EDHF has different vasoactive properties depending on the arteries studied [55,71]. Indeed, in rat mesenteric arteries the role of EDHF in relaxation appears to be increased during pregnancy [69].

To study the role of the AT1-R and AT2-R in the blunted responsiveness to Ang-II during pregnancy [16], we studied the in-vitro responsiveness of the AT1-R and AT2-R to Ang-II in the rat. The contractile response to Ang-II was dramatically decreased in P-saline infused rats as compared to the NP rats, which is in line with a decreased blood flow reducing effect of Ang-II during human pregnancy [16]. Our data also confirm previous studies in the rat [72,73]. Also, the increased contraction response to Ang-II in aortic rings of experimental preeclamptic rats as compared to P-saline infused rats appears to be in line with the well-known increased Ang-II sensitivity during human preeclampsia [19] and with studies in other models of experimental preeclampsia [74]. This increase in response to Ang-II may be caused by an increased AT1-R expression in LPS infused pregnant animals, although we only found a trend towards increase in AT1-R mRNA expression. Whether this increased response to Ang-II in the aorta of rats with experimental preeclampsia contributes to the hypertension seen in these animals, remains speculative, since the aorta is a conductance vessel and not a resistance vessel. Further studies into the response to Ang-II in other vessels on the preeclamptic rats are in progress. However, a role for Ang-II and the AT1-R in this model for experimental preeclampsia has been shown by Doering et al. who observed that hypertension was decreased in this model after treatment with the AT1-R antagonist losartan.

Interestingly, we found that the vasorelaxing response to Ang-II mediated through the AT2-R was absent during late pregnancy in the P-saline infused rat and increased in the P-LPS infused rats, suggesting that also the AT2-R does play a role in the adaptations of the sensitivity to Ang-II during normal pregnancy and preeclampsia. In contrast to the present study, however, Stennett et al. observed an increased responsiveness of the AT2-R to Ang-II during normal pregnancy [38]. The difference between our study and the study by Stennett et al. may be explained by strain differences, since Stennett et al. used Sprague-Dawley rats rather than Wistar rats. A recent review of van Drongelen et al., showed large differences in pregnancy induced vascular responses between Wistar and Sprague-Dawley rats [48] or concomitant long-term use of AT1-R blockers as shown in another study [75]. Apart from placental and uterine tissue [76–78], data on function and expression of the AT1-R and AT2-R in tissues of humans during pregnancy are largely lacking. Therefore, the role of the AT2-R versus the AT1-R during human pregnancy and preeclampsia is relatively unclear. Since

both the vasocontractory response (via the AT1-R) as well as relaxation response (via the AT2-R) to Ang-II were blunted at the end of pregnancy, but not in experimental preeclampsia, the RAAS may be of relative low importance in blood pressure control at the end of normal rat pregnancy, while in preeclamptic rats, the contribution of the RAAS may be enhanced.

Finally, our results show that the decreased involvement of EDHF in acetylcholine-induced relaxation during pregnancy correlates with a decreased relaxation responsiveness of the AT2-R to Ang-II in these conditions. This correlation may be in line with the suggestion that bradykinin and the B_2-receptor are involved in relaxation induced by Ang-II [79] and the notion that bradykinin-induced relaxation is typically mediated by multiple EDHF's [80]. Although NO and PG-F_2 are also suggested to play a role in the relaxation after binding of Ang-II to the AT2-R, we did not find a significant correlation between the role of NO or PG in the vasoactive capacity of the aorta and the AT2-R induced relaxation. This may suggest that the contribution of NO to relaxation was relatively constant and that differences in responses of the AT2-R to Ang-II during pregnancy and experimental preeclampsia may result from differences in the role of EDHF. The lack of correlation between the role of PG and the vasorelaxing effect of Ang-II may result from the fact that we did not specifically study PG-F_2, but PG in total.

Our observations were obtained with aorta which is a conductance vessel rather than a resistance vessel typically involved in blood pressure regulation. Our present observations in the aorta provide evidence of altered involvement of different endothelial mediators in acetylcholine-induced relaxation and responsiveness to Ang-II in pregnancy and preeclampsia. They need to be confirmed in future studies employing preparations of other vessels – such as small resistance arteries, for example – to more directly link changes in vascular function to hypertension in pregnancy. However, it may be speculated that such changes may play a role in the regulation of blood pressure by influencing vascular smooth muscle tone and therefore aortic stiffness and thus central blood pressure [81,82].It may be of additional interest in future studies to include the role of Ang 1-7 when aiming to unravel the RAAS pathways involved in the development of preeclampsia. Ang 1-7 is a metabolite of Ang-II which is able to counteract the vasoconstriction effect of Ang-II by binding to the MAS-receptor and subsequently causing vasodilation via NO through eNOS activation [83].

From this study, the pathway of how LPS induced the endothelial dysfunction and increased Ang-II seen in this experimental rat model for preeclampsia is still unknown. It is interesting to note that although the infusion of LPS took place on day 14 of pregnancy, endothelial dysfunction was observed on day 20 of pregnancy. This implies that LPS, which is infused during 1 hour on day 14, and cannot be traced in the circulation 15 minutes after infusion (unpublished data from previous work), induced a long-lasting effect in pregnant rats. This might be a direct effect of LPS on the endothelial cells, since LPS was shown to directly affect endothelial cells *in-vitro* [84]. However, it might also be an indirect effect, since it has been shown that LPS induced long-lasting activation of inflammatory cells in pregnant rats [26,30]. These activated inflammatory cells, for instance by producing oxygen free radicals, may then induce the endothelial cell dysfunction [85]. Future studies will be performed to test these two options.

In conclusion, pregnancy in the rat was associated with a change in the involvement of different mediators of thoracic aortic endothelial relaxation function and the response to Ang-II. The contribution of vasodilatory PG to acetylcholine-induced relaxation was increased while that of EDHF was decreased in pregnant rats, as compared to that in non-pregnant rats. Moreover, we observed a decreased sensitivity to Ang-II (both contraction and relaxation) in the aorta during pregnancy in the rat. Interestingly, the pregnancy-induced changes appeared to be absent in experimental preeclampsia in the rat. The present findings may imply that the LPS-induced pregnant rat is a suitable model for future studies aiming to unravel the etiology of preeclampsia and test treatment options directed to Ang-II receptors and endothelial cells in preeclampsia.

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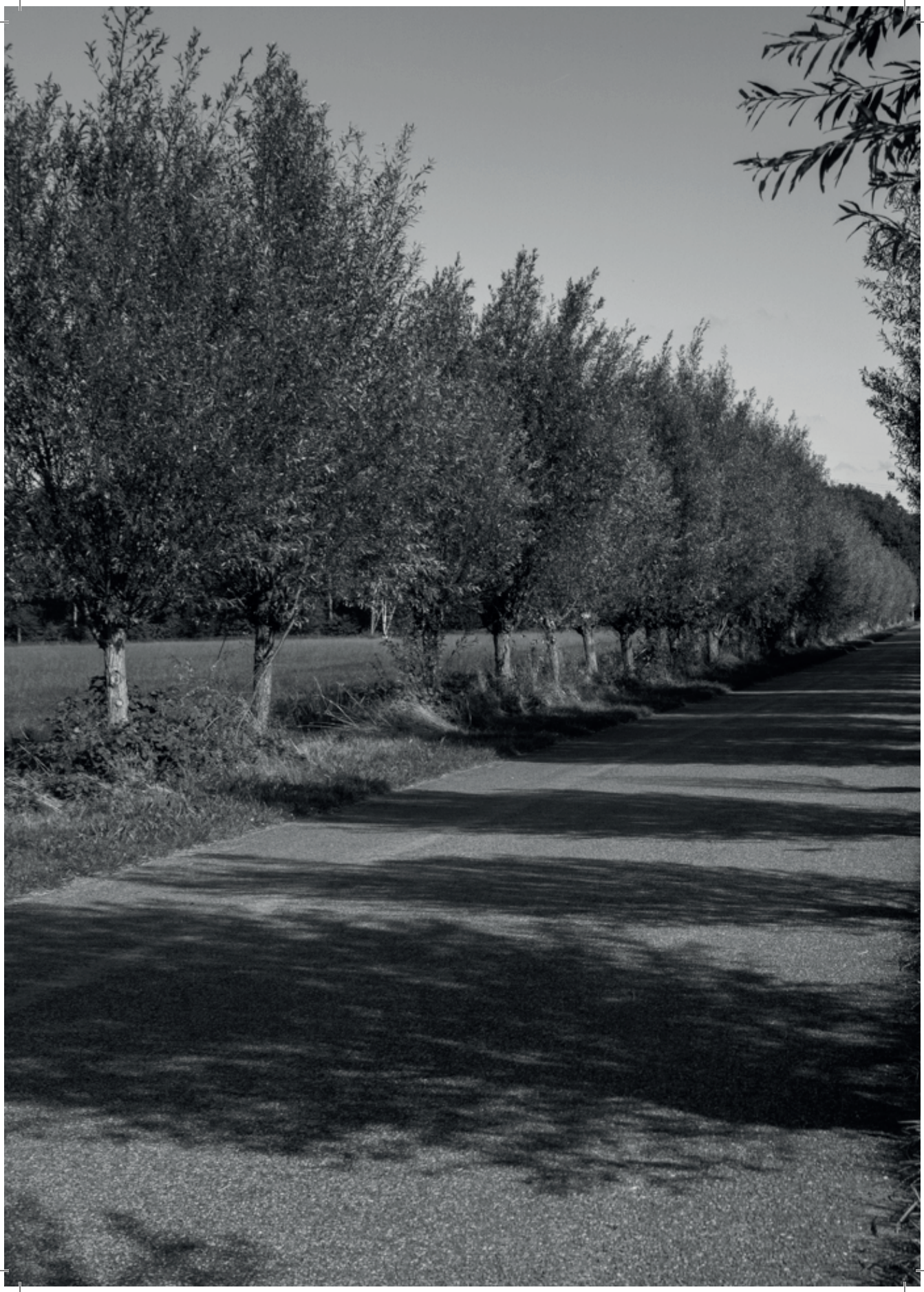

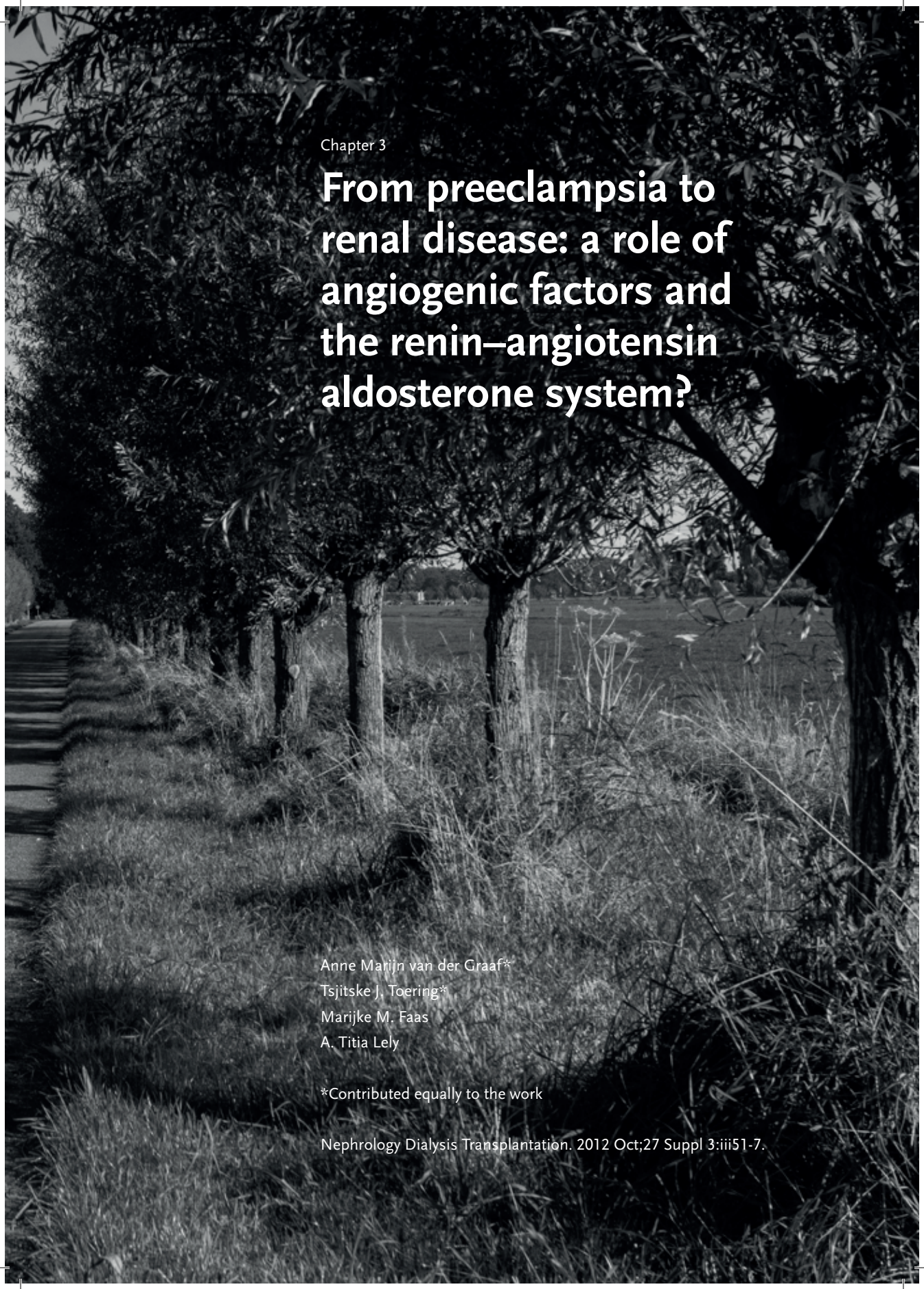

Chapter 3

From preeclampsia to renal disease: a role of angiogenic factors and the renin–angiotensin aldosterone system?

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ABSTRACT

Complicating up to 8% of pregnancies, preeclampsia is the most common glomerular disease worldwide and remains a leading cause of infant and maternal morbidity and mortality. Although, the exact pathogenesis of this syndrome of hypertension and proteinuria is still incomplete, a consistent line of evidence has identified an imbalance of proangiogenic and antiangiogenic proteins as a key factor in the development of preeclampsia [1–3]. Furthermore, recently, more attention has turned to the renin-angiotensin aldosterone system (RAAS) to provide understanding for the hypertension of preeclampsia [4]. The imbalance of the RAAS and imbalance between angiogenic and anti-angiogenic factors, which may be both common to preeclampsia and chronic kidney disease (CKD), might explain why a history of preeclampsia predisposes women to develop CKD.

In this review, we briefly describe the characteristics of preeclampsia with a focus on the mechanisms of angiogenesis and the RAAS and its role in the pathogenesis of preeclampsia. Our main focus will be on the intriguing association between preeclampsia and the subsequent increased risk of developing CKD and on the potential mechanisms by which the risk of CKD is elevated in women with a history of preeclampsia.

PREECLAMPSIA

The diagnosis of preeclampsia is clinical. As defined by the International Society of Studies of Hypertension in Pregnancy, the diagnosis requires blood pressures of 140/90 mmHg or higher on two occasions combined with urinary protein excretion of ≥300mg/day [5]. Laboratory tests, such as liver function tests, quantification of urinary protein, or serum creatinine may be helpful in characterizing the degree of end-organ damage, but none is specific for preeclampsia [6].

In the pathogenesis of preeclampsia, the placenta is the central organ since removal of the placenta abolishes the disease [7]. Pathological examination reveals several abnormalities including infarcts, atherosis, thrombosis, and chronic inflammation [8]. During normal placentation, the embryo-derived cytotrophoblast cells invade the maternal spiral arteries. As part of this process, the cytotrophoblasts adopt an endothelial phenotype [7,9]. In preeclampsia, the invasion of the cytotrophoblasts into the spiral arteries is incomplete; they are only present in the superficial layers of the decidua. The abnormal placentation is thought to lead to release of secreted factors that enter the mother's circulation, culminating in the clinical signs and symptoms of preeclampsia.

Kidney

Renal function undergoes physiological adaptations in pregnancy. Healthy pregnant women show marked glomerular hyperfiltration by 40 to 60% in the second half of pregnancy [10.] This hyperfiltration appears to result primarily from depression of the oncotic pressure [10]. Furthermore, an increased rate of effective renal plasma flow (ERPF) is found during pregnancy, (approximately 80% by 12 weeks' gestation) [10]. In contrast, during preeclampsia these functional changes in renal hemodynamics are different. The GFR in women with preeclampsia is significantly lower as compared with healthy gravid control subjects (91 ml/min per 1.73 m^2 vs 149 ml/min per 1.73 m^2). Of interest, no differences were found in ERPF between women with preeclampsia and healthy controls [11].

This depression in GFR during preeclampsia coincides with typical histopathological changes in the kidney, called glomerular endotheliosis, which is characterized by fibrin deposition, endothelial swelling and loss of capillary space [12]. Although these renal histological changes have been considered pathognomonic for preeclampsia, this may not be the case. Several groups have performed antenatal renal biopsies in normal pregnant women and women with gestational hypertension. For instance, Strevenset al. demonstrated that five of twelve normal pregnant women had, albeit very mild, evidence of glomerular endotheliosis [3]. These endotheliosis lesions resolve at variable rates postpartum, but it has been proposed that the characteristic renal changes of preeclampsia can be more long-standing [13]. The mechanism for hypofiltration during preeclampsia is not elucidated; both (renal) hemodynamic mechanisms and secondary changes to structural renal changes are proposed [4]. Lately, podocyte alterations and podocyturia have been described during preeclampsia [14]. Proteinuria in patients with preeclampsia might not only be mediated by endothelial alterations described classically, but also by disturbances of podocyte biology including impaired survival, enhanced apoptosis and down-regulation of nephrin and other key proteins of

the slit diaphragm [15].

Angiogenic factors

Recent observations support the hypothesis that altered expression of placental anti-angiogenic factors are partially responsible for the clinical manifestations of preeclampsia. Soluble Fms-like tyrosine kinase-1 (sFlt1, the soluble form of vascular endothelial growth factor (VEGF) receptor 1) and placenta-derived soluble TGF-B co-receptor endoglin (sEng), secreted by the placenta, are increased in the maternal circulation weeks before the onset of preeclampsia [3]. These elevated factors might lead to endothelial dysfunction and therefore decreased endothelium dependent vasodilation and proteinuria. Several days after delivery, sFlt1 levels normalize coinciding with decrease in proteinuria and blood pressure. The fact that increasing circulating sFlt1 levels in gravid mice and rats, either by direct infusion of the protein or by injecting adenovirus expressing the sFlt1 mRNA, produces a syndrome resembling human preeclampsia, including hypertension, proteinuria and glomerular endotheliosis, suggests that sFlt1 plays a role in the pathogenesis of preeclampsia [1,16].

The exact mechanism by which (anti)-angiogenic factors are involved in the development of the typical renal phenotype during preeclampsia is unknown. However, recent evidence shows that VEGF and its type 2 receptor have a clear role in the podocyte and slit diaphragm [17]. Overexpression of VEGF-A induces glomerular diseases [18]. VEGF produced by the podocyte regulates the structure and function of the adjacent endothelial cell.

Renin-angiotensin aldosterone system

In normal pregnancy there are marked changes in the RAAS including considerably elevated angiotensin II (ang II) levels [16]. However, vascular resistance decreases markedly during normal pregnancy, suggesting that pregnant individuals are less sensitive to ang II than non-pregnant individuals [19,20]. In contrast, during preeclampsia decreased circulating components of the RAAS with enhanced sensitivity to ang II infusion have been reported [5,20,21].

There are several possible explanations for this enhanced sensitivity of ang II infusion. Firstly, it may be mediated through increased placental ang II type 1 receptor (AT1-R) expression, which has been specifically observed to be upregulated on the decidual or maternal side of the placenta [22]. Secondly, angiotensin 1-7 (ang 1-7), a counterregulator of ang II, is decreased during preeclampsia compared with normal pregnancy. Therefore, this decreased ang 1-7 in these women may play a role in the hypertension seen in these patients [23]. A third explanation is that circulating ang II type 1 receptor autoantibodies (AT1-AA) levels are elevated, which may explain the hypersensitivity to the effects of ang II [24]. After injecting AT1-AA into pregnant mice and rats, hypertension, proteinuria, glomerular endothelial damage, and elevated levels of anti-angiogenic factors can be seen, suggesting that AT1-AA contribute to the pathogenesis of preeclampsia [25,26].

However, in humans, increases in AT1-AA are not specifically related to preeclampsia, as this autoantibody is also elevated during intra-uterine growth restriction without preeclampsia [27]. Therefore, the AT1-AA could be more related to impairment in placental development (placental ischemia) rather than mediating the preeclamptic phenotype. This is enforced by the fact that the

disappearance of clinical symptoms do not require the loss of the autoantibody [28].

Interaction between sFlt1 and RAAS

So far, most studies on preeclampsia have focused on angiogenic factors or on the RAAS, and not on the combination of both and the interaction between the two. Below, we will point out some evidence of interaction between sFlt1 and the RAAS.

A link between AT1-AA and angiogenic factors has been made by Zhou et al. [29]; circulating AT1-AA and ang II are capable of inducing sFlt1 production via AT1-R activation and downstream calcineurin/nuclear factor of activated T-cells signaling. Therefore, both may be responsible for placental sFlt1 up-regulation in preeclampsia. These observations extend a previous finding from the same group that ang II stimulates sFlt1 production in placental explants [30]. Moreover, Kim et al. have shown that in human proximal tubule cells, ang II induces an increased sFlt1 production cells, which is significantly blocked by losartan [31].

Furthermore, in pregnant mice, injection of serum from preeclamptic women with AT1-AA increases the sFlt1 concentrations via the AT1-R significantly as compared to pregnant mice injected with serum from normotensive pregnant women [29]. Moreover, another study in mice shows that the AT1-AA induced hypertension, proteinuria and renal abnormalities is reduced by co-injection with losartan or infusion of VEGF121 [32]. In contrast, in the transgenic renin overexpression preeclampsia rat model, in which AT1-AA is elevated, no increase in sFlt1 concentration was found [33].

Whether a similar relation between AT1-AA and sFlt1 also exist in human pregnancy is unclear. In preeclamptic patients, some studies show no correlation between sFlt1 and AT1-AAs [34,35], while another study shows the titer of AT1-AA, is significantly correlated with sFlt1 level in severe preeclamptic women [36].

In conclusion, although the exact interaction between the RAAS and sFlt is still unknown (especially in humans), the studies described above suggest that during preeclampsia the impairment in the RAAS may influence the sFlt1 production.

AFTER PREECLAMPSIA

Long-term vascular risk after preeclampsia

Long term vascular risk following a preeclamptic pregnancy has been recognized for many years. Already in the 1960s [36] it was shown that a history of preeclampsia increases the risk of future hypertension. A meta-analysis showed that 1885 of 3658 formerly preeclamptic women developed chronic hypertension in a follow-up time of 5-32 years. Therefore, the relative risk of a later diagnosis of hypertension in formerly preeclamptic women is 3.7 [37]. Not only the risk for hypertension is increased in formerly preeclamptic women, they also have a higher risk for hypertension related diseases, i.e. ischaemic heart disease, in the next 15-19 years [38]. Furthermore, another meta-analysis concluded that formerly preeclamptic women have an approximately double risk of early cardiac, cerebrovascular, and peripheral arterial disease, and cardiovascular mortality [39].

Kidney disease after preeclampsia

Preeclampsia is more common in women with an underlying kidney disease. On the other hand, it has been suggested that preeclampsia itself increases the risk of kidney disease later in life. From the early 1960s till 1980s, several renal biopsy studies discussed the appearance of renal damage in women who had a pregnancy affected by preeclampsia. Zech et al. have studied the appearance of renal injury in biopsies three months to four years after pregnancy in a series of sixty formerly preeclamptic women. In many cases, typical glomerular lesions disappeared rapidly within six months. However, vascular lesions remained the same or changed only very slowly. Furthermore, these vascular lesions were directly related with an increased blood pressure [40].

Although nowadays, postpartum renal biopsy studies in formerly preeclamptic women are uncommon, a recent large cohort study has shown that a history of preeclampsia is a risk factor for undergoing a kidney biopsy later in life [41] . In a cohort of 756,420 women, the relative risk of undergoing a kidney biopsy was significantly higher in women with a history of preeclampsia (RR of 12 (95% CI 6.3 to 23)). Subsequently, in another large cohort of 570,433 women, Vikse et al. studied the risk of developing end stage renal disease (ESRD) after preeclampsia [42] Preeclampsia during the first pregnancy was associated with a relative risk of ESRD of 4.7 (95% CI 3.6 to 6.1). Moreover, if women also had preeclampsia during subsequent pregnancies the relative risk of ESRD was even higher (15.5 95% CI 7.8 to 30.8). According to the authors, the association was stronger in preeclamptic women who had given birth to preterm infant or child with low birth weight, which might suggest that women with severe, early-onset preeclampsia have a higher risk for developing ESRD.

Microalbuminuria after preeclampsia

A recent meta-analysis concluded that 31% of formerly preeclamptic women had microalbuminuria compared with 7% of women with uncomplicated pregnancies on average 7.1 years postpartum. This prevalence is similar to that found in patients with type 1 diabetes mellitus (28% at 14 years after diagnosis) [43]. Furthermore, women have a 4-fold increased risk of microalbuminuria after mild preeclampsia, and a 8-fold increased risk after severe preeclampsia, suggesting a graded relationship according to severity of the preeclamptic episode [43]. The high incidence of microalbuminuria in formerly preeclamptic women found in the meta-analysis might partly be caused by the inclusion of a study that examined women with diabetes mellitus type 1 [44]. Especially since diabetic nephropathy, which is present in 41.9% of the formerly preeclamptic women with diabetes, is associated with elevated blood pressure and endothelial dysfunction. Still, the finding that almost one-third of formerly preeclamptic women have microalbuminuria is important given the associated risks of ESRD and cardiovascular disease. Several factors may explain the increased risk of microalbuminuria in formerly preeclamptic women: undetected hypertension and microalbuminaria before pregnancy, shared risk factors for preeclampsia and kidney disease and the fact that preeclampsia itself may damage the kidneys with scarring or incomplete healing from the endotheliosis. A potential mechanism of long-term renal damage might be that renal damage during preeclampsia results in epigenetic changes, and therefore leading to permanent kidney

damage. It would be interesting to unravel the role of epigenetic changes in the development of kidney damage after preeclampsia.

Renal function parameters after preeclampsia

Although several studies, as described above, have found persistent microalbuminuria in formerly preeclamptic women several years after pregnancy, no differences were found in serum renal function parameters in formerly preeclamptic women [43]. None of these studies have shown a significant difference in serum creatinine values or creatinine clearance between formerly preeclamptic women and healthy parous controls. However, when looking at particular characteristics of renal function and performing renal function measurements in a more accurate way, abnormalities in renal function in formerly preeclamptic women can be seen.

In 1998, van Beek et al. examined renal hemodynamic values (ERPF and GFR) at least four months postpartum in primiparous formerly preeclamptic women and healthy parous controls [45]. These renal hemodynamic values were derived from the continuous infusion of para-aminohippurate and inulin. Although, GFR did not differ significantly between the groups, ERPF appeared to be significantly lower in formerly preeclamptic women (482 ± 88 vs 553 ± 67 ml/min/1.73m^2). Consequently, filtration fraction (FF) was significantly higher in formerly preeclamptic women (0.28% ± 0.04% vs 0.22% ± 0.03%). Likewise, renal vascular resistance (RVR) was also higher.

More recently, Spaan et al. compared in a similar protocol formerly preeclamptic women with parous controls at least 20 years after pregnancy [46]. They also found a significant difference in ERPF (399 ± 61 vs 463 ± 83 mL/min/1.73m^2 former preeclamptic women vs controls respectively) and RVR (122 ± 28 vs 95 ± 20). Furthermore, creatinine clearance appeared to be significantly lower in formerly preeclamptic women (88 ± 15 vs 100 ± 19 mL/min/1.73m^2).

According to these results, we can conclude that women who suffered from preeclampsia have abnormalities in renal function later in life. Both after short- and long-term follow-up an increased RVR and a reduced ERPF is observed, which supports the idea that renal vasoconstriction might play a central role in the development of hypertension and renal impairment in formerly preeclamptic women.

Renin-angiotensin aldosterone system after preeclampsia

During preeclampsia the plasma levels of ang II are decreased, while the sensitivity for ang II is increased [5,20–22]. In most cases, circulating ang II levels normalize within three months after delivery [5,20–22]. However, in one study, upregulation of ang II was found in hypertensive subgroups of formerly preeclamptic women [5,20–22]. Although there is no clear difference between formerly preeclamptic women and healthy controls in circulating ang II levels, several studies have suggested a difference in ang II sensitivity.

Three studies have examined the ang II sensitivity in formerly preeclamptic women [47–49]. One study showed no difference in blood pressure response to ang II between formerly preeclamptic women and healthy controls [50]. However, the two other studies conducted ang II infusion both during low and high sodium diet, and showed increased blood pressure responsiveness to

ang II during low sodium intake in women with a history of gestational hypertension or preeclampsia [47,49]. Furthermore, Hladunewich et al. have shown that after stimulating the RAAS with lower-body negative pressure, a delayed increase in circulating RAAS components was found in formerly preeclamptic women [49].

Differences in results in the studies mentioned above can be explained by the use of different doses of ang II, whether or not using a standardized sodium diet and studying patients at different periods of the menstrual cycle [51]. Sodium intake is of major influence on the RAAS and ang II sensitivity, since, low sodium diet is a strong activator of the RAAS. From the above studies it seems that there are differences in the function of the RAAS in formerly preeclamptic women as compared with women who experienced a normal pregnancy. However, this may only be revealed under certain circumstances, such as under low salt diet. In this respect it is of interest to mention that after pregnancy complicated by hypertension increased pressor response to a high sodium diet, e.g. sodium sensitivity is reported [47]. This increased sodium sensitivity could lead to renal disease on the long term.

As mentioned above, a different expression of AT1-R may be involved in the enhanced ang II sensitivity found in formerly preeclamptic women. However, Hladunewich et al. could not show differences in AT1-R mRNA expression on tissue level in skin biopsies [49]. Another explanation for the enhanced ang II sensitivity is elevation of AT1-AA levels in formerly preeclamptic women. Although AT1-AA levels decline by 50% at one week after delivery, Hubel et al. concluded that significantly more formerly preeclamptic women were characterized by the presence of AT1-AA as compared to healthy controls (17.2% vs 2.9%) [52]. Because ang II increases sFlt1 production in the human proximal tubule cell, the existence of AT1-AA and increased levels of sFlt1 may be linked [53].

Concerning other RAAS parameters, no differences in plasma renin concentration and plasma aldosterone concentrations were found between former preeclamptic women and control pregnancies at least four months postpartum [45,48,54,55]. However, in response to ang II infusion, aldosterone levels in women with a history of new-onset hypertension during pregnancy are significantly increased as compared to normotensive controls [47]. Another study could not confirm this increased aldosterone response [48]. Again, the difference in results might be explained by the differences in concentration and duration of ang II infusion and sodium intake.

sFlt1 after preeclampsia

Even though sFlt1 levels rapidly decrease during the first week after delivery, indicating that the large fraction of sFlt1 is derived from the placenta, increased levels of sFlt1 have been found in formerly preeclamptic women [52,56,57]. This persistence of increased levels of sFlt1 in formerly preeclamptic women might be due to a richer extra-placental source of sFlt1 in these women, such as endothelial cells and monocytes. Indeed, one year postpartum, formerly preeclamptic women showed increased expression of sFlt1 in peripheral blood mononuclear cells [58]. This increased sFlt1 may lead to changes in the vascular endothelium which may increase the risk of reno-vascular diseases in later life. Interestingly, patients with CKD and decreased GFR without a history of preeclampsia present with increased plasma levels of sFlt1, which correlates positively with proteinuria [59].

However, other studies did not find a significant difference in baseline sFlt1 levels between women with a history of new-onset hypertension in pregnancy and women with a history of normotensive pregnancies [2,47,60,61]. Interestingly, in one of these studies it is shown that during low-sodium balance, women with a history of gestational hypertension had greater sFlt1 response to ang II compared to women with a history of normotensive pregnancy [47]. This not only reinforces an interaction between the RAAS and sFlt1, as described above, but also shows that it is important to take sodium status into account when studying the interaction between RAAS and angiogenic factors. It also shows that sodium intake could be a factor of influence in the discrepancy between the human studies on the relationship between AT1-AA and sFlt1 as described above. This interaction between sFlt1 and the RAAS after preeclampsia is interesting and should be elucidated in future studies.

CONCLUSIVE REMARKS

In summary, preeclampsia is a risk factor for the development of CKD. Why preeclampsia is associated with an increased risk for developing kidney disease is still unclear. One possibility could be that both preeclampsia and CKD are caused by the same risk factors (such as hypertension, obesity, insulin resistance, and endothelial dysfunction). Furthermore, the role of angiogenic factors (sFlt1) and the RAAS (AT1-AA and up-regulation of AT1-R) in the pathophysiology of both preeclampsia and CKD reinforces the described association. In regards to well-known cardiovascular risk factors, Romundstad et al have shown that approximately 50% of the elevated risk for future hypertension after preeclampsia can be explained by preexisting risk factors [62]. Therefore, as it has been described by Sattar et al., pregnancy might be a stress test that can reveal subclinical cardiovascular and renal disease [63].

Moreover, preeclampsia itself induces deleterious effects to the kidney, revealed by persistent microalbuminuria in a subset of formerly preeclamptic women. As described above, during preeclampsia and still after preeclampsia, women have a disturbance in their regulation of the RAAS (increased sensitivity for angII, possible by up-regulation of the AT1-R and increased AT1-AA). This might induce persistent increased ang II sensitivity, increased salt sensitivity and altered renal hemodynamics. In addition, preeclampsia induced endothelial dysfunction appears to remain after preeclampsia in a lot of patients [64] and is an independent risk factor for future cardiovascular and renal health. The reason for the remaining endothelial dysfunction is unknown, it may, however, be suggested that preeclampsia induces epigenetic changes in endothelial cells. Endothelial cell dysfunction may also be enhanced by increased levels of sFlt1 in formerly preeclamptic women. Ultimately, these disturbances in formerly preeclamptic women lead to an increased risk for CKD (figure 1). Future research should attempt to elucidate the exact mechanisms that underlie the complex interaction between preeclampsia and kidney disease.

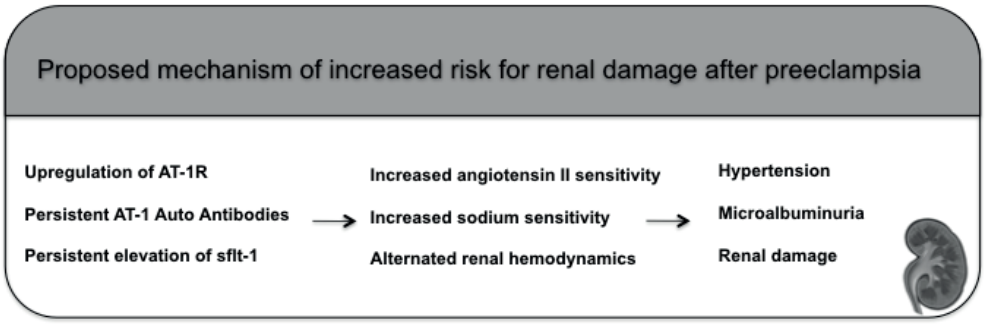

Figure 1. Proposed mechanisms.

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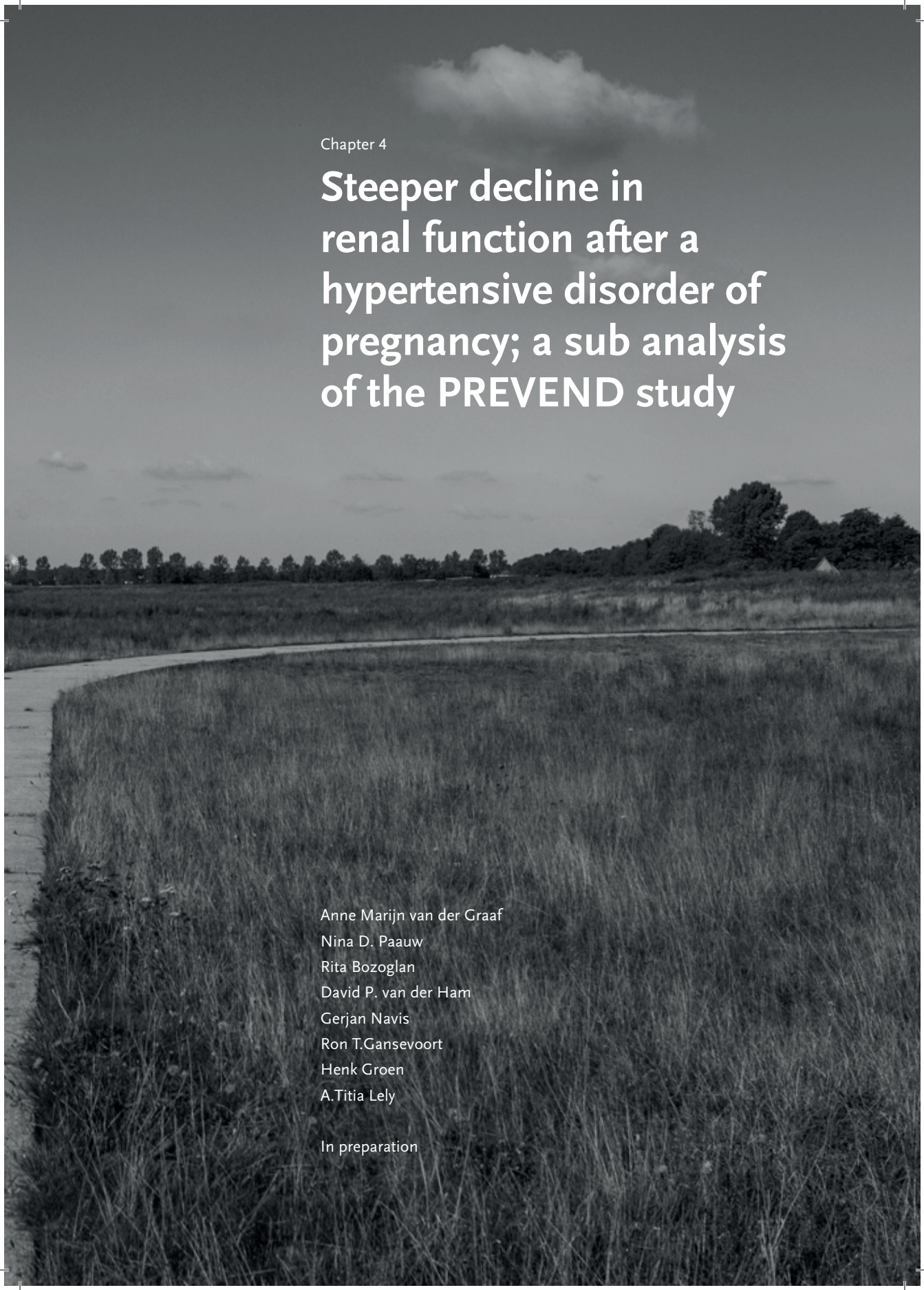

Chapter 4

Steeper decline in renal function after a hypertensive disorder of pregnancy; a sub analysis of the PREVEND study

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ABSTRACT

Introduction

Hypertensive disorders of pregnancy (HDP) complicate about 10% of pregnancies. Population based studies report an increased risk for developing end stage renal disease after HDP. So far, studies have reported on renal function early after HDP. Longitudinal renal function and occurrence of chronic kidney disease (CKD) after HDP have not been studied yet.

Aim

The aim of this study was to longitudinally assess postpartum renal function and occurrence of CKD in women with a history of HDP compared to controls.

Materials and Methods

We followed two approaches. First, in the PREVEND study, a general population based prospective cohort study, we identified women without and with a history of a self-reported HDP (non-HDP, n=1805 and HDP, n=977; respectively). Mean age at start of the study was 50 ± 12 years and median follow-up time was 11 years. Second, in a subgroup where hospital records on pregnancy history could be retrieved, we created a cohort 10 years postpartum specified by class of hypertensive disorder, namely normal pregnancy (control, n=202), pregnancy-induced hypertension (PIH, n=56) or preeclampsia (PE) or haemolysis elevated liver enzymes and low platelets (HELLP) syndrome (PE/HELLP, n=29). Renal function and the prevalence of CKD (defined as: eGFR <60ml/min per 1.73m^2 and/or 24-hour albuminuria >30mg) were assessed in both approaches.

Results

At baseline and during follow-up eGFR was lower in HDP (98.0 ± 16.2) vs non-HDP (99.4 ± 16.7) (p=0.03). In addition, the decline in eGFR was significantly steeper in HDP (p for interaction=0.04). At baseline and during follow-up there was more anti-hypertensive-drug use in the HDP group. 24-hour albuminuria was higher in HDP and remained so during follow-up. Hazard ratio (95% confidence interval (CI)) for development of CKD during follow-up was 1.13 (0.92-1.38; p=0.24) in HDP. In the sub-group analysis, eGFR was significantly lower and 24-hour albuminuria significantly higher in the PE/HELLP as compared to controls (p=0.01 and p=0.03; respectively). CKD was present in 7.8% of the controls, 10.9% of the PIH group and 19.2% of the PE/HELLP group. The odds-ratio and 95% CI for CKD risk for PE/HELLP women with controls as reference group was 1.76 (0.93-3.34; p=0.08).

Conclusion

We report a slightly but significantly lower renal function and a steeper renal function decline over time after HDP. Women with severe hypertensive disorders of pregnancy (PE/HELLP) have the highest risk of CKD.

INTRODUCTION

Hypertensive disorders of pregnancy (HDP) occur in up to 10% of all pregnancies and are a major cause of maternal morbidity and mortality worldwide [1]. The most severe form of HDP is preeclampsia (PE), clinically characterized by the presence of hypertension in combination with proteinuria in the second half of pregnancy [2]. PE can be accompanied by the HELLP syndrome (haemolysis, elevated liver enzymes and low platelets), renal failure, and eclampsia (seizures).

In the last 5-10 years it was shown that women with a history of HDP have an increased risk of end stage renal disease (ESRD). Vikse et al. were the first to show that formerly preeclamptic women have a relative risk of 4.7 for developing ESRD [3]. This risk triples in case of recurrent PE or when complicated by low-birth-weight or premature delivery [3]. Later, two Taiwanese cohort studies (with overlap) found a HR of 10.6-14 for developing ESRD after PE [4, 5]. One of the Taiwanese cohorts found that women with a milder form of HDP (pregnancy induced hypertension (PIH)) also have an increased risk to develop ESRD with a HR of 5.8 [4]. The exact risk of chronic kidney disease (CKD) in formerly preeclamptic women is unknown, but Wang et al. estimate the risk for CKD to be 4 to 10-fold higher in women with a history of HDP [5].

Long-term accelerated renal function decline might underlie the increased risk for ESRD in formerly preeclamptic women. However, studies assessing renal function shortly after HDP reported either subtle alterations or no differences in renal function and renal hemodynamics [6-10]. Up until now, there are no studies reporting long-term renal function, renal function loss over time and CKD risk after HDP.

In order to evaluate long-term renal function, renal function decline over time, and the occurrence of CKD after HDP, we used data from the PREVEND study [11]. The PREVEND study is a general population based prospective observational cohort study in the city of Groningen with longitudinal data on kidney function and albuminuria, which included questionnaires assessing self-reported HDP. Data on five different time points, over a maximum follow-up of fourteen years are presented. In addition, to study the effect of severity of the HDP on renal function we performed record linkage with hospital records on pregnancy history in the subgroup of women that gave birth in one of the Groningen hospitals. Thus we created a case-control cohort at 10-years postpartum, including women with history of a healthy pregnancy, pregnancy induced hypertension (PIH) or HELLP/PE. We hypothesize that a history of an HDP increases the risk for accelerated long-term renal function loss and hence the risk to develop CKD.

MATERIALS AND METHODS

PREVEND study

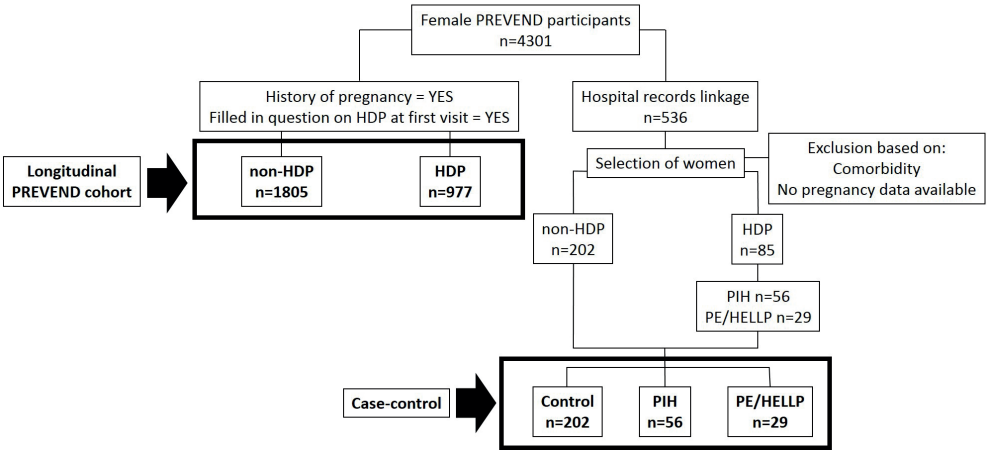
The PREVEND study is a prospective investigation of renal function, albuminuria, renal, and cardiovascular disease in a large cohort drawn from the general population [12]. Details of this study are described elsewhere [13, 14]. In summary, from 1997 to 1998, all inhabitants of Groningen, the Netherlands, aged 28–75 years (n=85,421), were sent a questionnaire and a vial to collect a first-morning void urine sample. Pregnant women and subjects with type 1 diabetes mellitus were excluded. The urinary albumin concentration was assessed in 40,856 responders. Subjects with a urinary albumin concentration ≥10mg/L (n=7,768) were invited to participate, of whom 6000 were enrolled. In addition, a randomly selected group with a urinary albumin concentration <10 mg/l (n=3,394) was invited to participate in the cohort, of whom 2592 were enrolled. From the period 1997-2012, five screening moments took place (around every 3 years) and detailed measurements were performed and blood samples were drawn and 24-hour urine was collected on two consecutive days. The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen. Written informed consent was obtained from all participants. These 8,592 individuals form the PREVEND cohort.

Selection of subjects

In total, 4,301 women were included in the PREVEND cohort (see Flow chart). Women who ever reported a pregnancy and answered the question regarding hypertension during pregnancy at the first visit were included in our study (n=2,782). The question on whether the women experienced hypertension during their pregnancy could be answered by the following options, 'no', 'I don't know', 'yes, but allowed to do anything' and 'yes, had to keep bed rest'. Women, who had not reported hypertension during pregnancy at the first visit, were categorized as 'women with no self-reported hypertensive pregnancy disorder' (non-HDP; n=1,805). Women who had reported 'yes, allowed to do anything' or 'yes, had to keep bed rest' at the first visit, were categorized as 'women with a self-reported hypertensive pregnancy disorder' (HDP; n=977).

Data collection

Values for systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), levels of urine albumin, serum creatinine, and serum cystatin C during the five visits were used for current analysis. Measurement of urinary albumin, serum creatinine, and serum cystatin C were performed as previously described [12]. 24-hour albuminuria was calculated with the formula Urinary Albumin Concentration (mg/L) x Urinary Volume (L) over 24-hours. Since participants collected 24-hour urine on two consecutive days, the mean of two 24-hour albuminuria levels was taken. The eGFR was calculated with the CKD-EPI creatinine–cystatin C equations, using the serum creatinine and serum cystatin C values [15]. The outcome of CKD was defined as a combination of an eGFR <60ml/min per 1.73m^2 and/or 24-hour albuminuria >30mg [12]. The development of CKD was calculated for each visit separately.



Flow chart of the patient selection for the PREVEND cohort and the case-control.

n: number of subjects; HDP: hypertensive disorder of pregnancy; PIH: pregnancy induced hypertension; PE: preeclampsia; HELLP: haemolysis elevated liver enzymes low platelets syndrome

The occurrence of ESRD was defined as women with a history of dialysis and or kidney transplantation (self-reported dialysis at visit 1 and during follow-up by linking with dialysis/kidney transplantation national database). Alcohol use was defined as 2-7 glasses a day or more. To calculate the percentage of women using anti-hypertensive medication, angiotensin converting enzyme inhibitors (ACE-i), and angiotensin receptor blockers (ARB) during follow-up, prescription data from pharmacies was collected. Oral contraceptive (OCC) use was based on self-reported use for any reason ('contraception', 'climacterium' or 'other reason'). Cardiovascular comorbidity includes a history of chronic heart disease or cerebrovascular accident. Homeostatic model assessment index was calculated using the formula (glucose*insulin)/22.5.

The PREVEND database could not provide information on delivery dates; therefore no postpartum follow-up time was calculated. Consequently, the date of the first PREVEND visit was taken as time-point '0'. The median follow-up time at each visit was calculated from time-point '0' where after follow-up time was divided in 4, 6, 9, and 11 years of follow-up. The maximum follow-up time was 14 years.

Case-control study in subgroup with obstetric history available

In order to relate renal function to the severity of the HDP an additional case-control study was performed. The PREVEND database had only data on self-reported hypertension and no specific details on the obstetric history. To obtain data on obstetric history, the PREVEND database was linked to the registry of the Obstetric departments from the two hospitals in the city of Groningen (the University Medical Centre Groningen and the Martini Hospital Groningen). Data from women who gave birth between 1991 and 2007 could be retrieved. Using a unique patient number or using surname combined with date of birth, this case-control data file could be merged with the PREVEND database. Within the time frame studied, 536 women that gave birth in one of the two Groningen

hospitals could be identified. The linking process resulted in a cohort with detailed information regarding the pregnancies, severity of the hypertensive disorder (PIH, PE, and HELLP-syndrome), delivery dates, highest DBP, proteinuria, and birth weight of the child. Postpartum follow-up time could be calculated by the use of the delivery data collected from the medical records. For this subgroup analysis the renal function parameters were used and compared 10 years postpartum.

Selection of cases

From the data of the 536 women that could be retrieved via record linkage, highest DBP measured during pregnancy was used to select women who fulfilled the criteria of a hypertensive disorder of pregnancy (DBP ≥ 90 mmHg). Subsequently, data regarding proteinuria was checked to identify the former preeclamptic women. Laboratory results, medical letters, electronic health records, and paper medical records were used to check for proteinuria. Additionally, laboratory evidence of thrombocyte count of ≤100x10^9/L, aspartate aminotransferase level of ≥50 IU/L, alanine aminotransferase level of ≥50 IU/L, and lactate dehydrogenase ≥250 IU/L was investigated to identify women with a history of HELLP syndrome. Thus, we classified women as: history of pregnancy-induced hypertension (PIH; n= 56) or: history of PE and/or HELLP syndrome (PE/HELLP; n=29). PIH was defined as a highest DBP ≥90 mmHg. PE was defined as a highest DBP of ≥90 mmHg and proteinuria (≥300 mg/24h or a dipstick label '> once ++ or once +++' or ' > once +++ or >0,5 gr/L' or '>3 times + or once ++'). Some women had a history of both PIH and PE in different pregnancies. In these cases, women were allocated to the group with PE/HELLP. Some women had a history of more than one hypertensive pregnancy. In that case, the pregnancy with the longest follow-up duration was selected.

Selection of controls

Women with a history of normotensive pregnancy (DBP <85 mmHg), birth weight of the newborn ≥2500 gram and no history of placental abruption were selected (n=319) and served as control group. Medical codes and electronic health records were checked to exclude women with any relevant comorbidity. All women with a history of diabetes mellitus, cardiovascular and renal disease were excluded. Cases of preterm birth, dysmaturity and perinatal mortality were also excluded. Since controls were randomly sampled, matching was not performed. In total, 202 controls were selected.

Statistical analysis

Data were analysed using SPSS 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad prism 5.01 (GraphPad Software Inc. San Diego, CA, USA). Parametric data are presented as mean ± standard deviation (SD) or 95% confidence interval (CI) in text, tables and figures and analysed using Student t-test or One-Way ANOVA followed by LSD post-hoc analysis. Non-parametric data are expressed as median with 25th-75th percentile and analysed using Mann-Whitney U test. The Chi-square test and Linear-by-Linear association were used for categorical variables. To assess the validity of maternally self-reported hypertension during pregnancy sensitivity and specificity was calculated. Sensitivity was calculated as the percentage true hypertensive women (PIH and PE/HELLP) according to the medical record reported as hypertensive in the questionnaire. Specificity was calculated as the

percentage true non-hypertensive women (Control) reported as no hypertension during pregnancy. For comparison of the eGFR decline during follow-up (factor visit) and the effect of a history of HDP or non-HDP (factor group), a generalized estimating equations (GEE) analysis was performed with the interaction term group*visit and corrected for BMI and age. In order to correct for ACE-i and ARB use on eGFR decline between the groups, an interaction term between ACE-i and ARB use and group was added in the GEE analysis. Exchangeable was used as correlation matrix and data are presented as Estimated Marginal Means (EMM) ± Standard Error (SE). Since a lower eGFR value is a risk factor for renal function decline over time in itself, GEE analysis was not corrected for eGFR at time-point 0. GEE-analysis was also used for comparing anti-hypertensive medication, ACE-i and ARB use, and the development of CKD during follow-up. To examine the prospective association between the risk of CKD and a history of HDP, Cox proportional hazard regression model was performed to calculate the hazard ratio (HR) and 95% confidence interval (CI) with non-HDP as the reference group. In this proportional hazard regression model, person time was counted from the date of the first visit until the date that CKD was diagnosed, or the date of the last visit, whichever came first. No covariates were added into the model. Data from the first PREVEND visit were used as baseline data for the case-control sub-study. To evaluate 10 years postpartum risk for the development of CKD in our case-control sub-study, logistic regression analysis was performed to calculate the odds ratio (OR) and 95% CI with BMI and age correction. In all analysis, a p-value <0.05 was considered significant.

RESULTS

PREVEND study – baseline characteristics

Table 1 shows the characteristics of the PREVEND population at time point 0. At baseline, women were on average 50 years old. Ethnicity, BMI, blood pressure, working status, smoking, anti-hypertensive, ACE-i, ARB, and OCC medication use, and cardiovascular comorbidity were significantly different between non-HDP and HDP. Laboratory cardiovascular risk markers were all significantly increased in HDP vs non-HDP at baseline, eGFR was slightly but significantly lower, and 24-hour albuminuria significantly higher in HDP vs non-HDP. No significant difference was found in the percentage of women that required dialysis.

PREVEND study – blood pressure

Although average blood pressure was within the normotensive range in both groups, SBP and DBP were significantly higher in HDP than in non-HDP at baseline (Table 1) and during follow-up (Figure 1A). During follow-up a rise in MAP was observed in the non-HDP group, while there was a trend towards an initial decrease and afterwards increase in the HDP group (possible treatment effect, p for interaction = 0.04; Figure 1A). The percentage of ACE-i, ARB, and all-anti-hypertensive use during follow-up increased significantly in HDP compared to non-HDP women (p for interaction<0.01 and p=0.03 respectively, Figure 1B/C).

Table 1. Baseline characteristics of the PREVEND population

	No hypertensive disorder of pregnancy (N=1805)	Hypertensive disorder of pregnancy (N=977)	P
Age (years)	50.1 ± 12	51.0 ± 11	0.05
Caucasian (n (%))	1700 (94.9)	940 (97.2)	<0.01
BMI (kg/m²)	25.8 ± 4.5	27.4 ± 5.0	<0.001
SBP (mmHg)	122 ± 20	130 ± 22	<0.001
DBP (mmHg)	70 ± 9	74 ± 9	<0.001
Birth weight (gram)	3281 ± 613	3283 ± 599	0.95
Job (n (%))	724 (40.9)	355 (37.0)	<0.01
Current smoker (n (%))	641 (35.5)	301 (30.8)	<0.05
Alcohol use (n (%))	802 (44.6)	449 (46.1)	0.44
Antihypertensive use (n (%))	181 (11.5)	209 (24.2)	<0.001
ACE-inhibitor use (n(%))	48 (3.0)	68 (7.8)	<0.001
Oral contraceptive use (n(%))	407 (24.2)	182 (20.4)	<0.05
Gestational diabetes (n (%))	40 (2.2)	20 (2.0)	0.77
Cardiovascular comorbidity (n (%))	42 (2.4)	36 (3.8)	<0.05
Medical history (n (%))			
Asthma/COPD	174 (9.6)	91 (9.3)	0.78
Thyroid disorders	87 (4.8)	54 (5.5)	0.42
Arthritis	126 (7.0)	74 (7.6)	0.56
Epilepsy	10 (0.6)	6 (0.6)	0.84
Laboratory results			
Glucose (mmol/l)	4.6 (4.2-5.0)	4.7 (4.3-5.1)	<0.001
Insulin (mmol/l)	7.4 (5.2-10.7)	8.3 (5.9-12.5)	<0.001
HOMA	1.5 (1.0-2.3)	1.7 (1.2-2.8)	<0.001

	No hypertensive disorder of pregnancy (N=1805)	Hypertensive disorder of pregnancy (N=977)	P
Cholesterol (mmol/l)	5.5 (4.8-6.4)	5.7 (5.0-6.5)	<0.01
Uric acid (mmol/l)	0.25 (0.22-0.30)	0.27 (0.23-0.31)	<0.001
Triglycerides (mmol/l)	1.06 (0.8-1.5)	1.13 (0.9-1.6)	<0.001
Renal function			
Creatinine (mmol/L)	63 ± 13	64 ± 12	0.30
Cystatin C (mg/L)	0.86 ± 0.17	0.87 ± 0.16	0.18
Renal disease requiring dialysis (n (%))	7 (0.4)	1 (0.1)	0.18

Data are presented as mean ± SD or median (25^{th} – 75^{th} percentile) unless otherwise stated. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; ACE: angiotensin converting enzyme; COPD: chronic obstructive pulmonary disease; HOMA: homeostatic model assessment index; eGFR: estimated glomerular filtration rate.

PREVEND study – renal outcomes

Average values for eGFR during follow-up were within the normal range in both groups (Figure 2A), with, however slightly but significantly lower values in HDP as compared to non-HDP at all visits. Moreover, the decline in eGFR was significantly steeper in the HDP compared to the non-HDP (p for interaction=0.04; Figure 2A). Moreover, from 6 years on into follow-up, the percentage decrease in eGFR (eGFR at visit 1 served as reference) was significantly higher in HDP vs non-HDP (p for interaction=0.01; Figure 2B). Although significantly more HDP as compared to non-HDP were using ACE-i/ARB at baseline (Table 1) and ACE-i/ARB use had a significant effect on eGFR during follow-up (lower eGFR in women on ACE-i/ARB, p=0.008), the decline in eGFR was still significantly steeper in HDP compared to non-HDP after correcting the GEE-analysis for ACE-i/ARB use (p for interaction=0.02). 24-hour albuminuria was significantly higher in HDP compared to non-HDP up to 6 years follow-up, were a trend was seen from 9 years on into follow-up (Figure 2C). During the complete follow-up, no significant interaction was found between group (HDP and non-HDP) and follow-up duration for 24-hour albuminuria (p for interaction=0.09; Figure 2C).

During follow-up, a total of 407 women developed CKD. The percentage of women developing CKD during follow-up showed a trend towards an increased percentage in HDP women as compared to non-HPDs women (p_{group}=0.09), without an interaction between group and visit (p for interaction=0.57). Compared with non-HDP the HR and 95% CI for CKD risk was 1.13 (0.92-1.38; p=0.24) for HDP women. During follow-up, no women developed ESRD in either group.

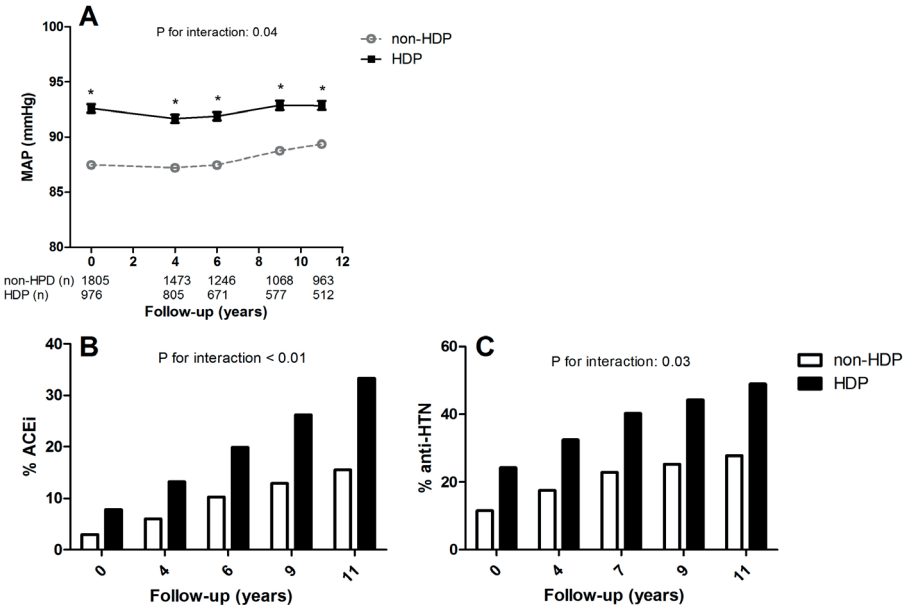


Figure 1. Prospective data on blood pressure and anti-hypertensive medication use in PREVEND cohort.

*The estimate marginal means (EMM) ± standard error (SE) of the mean arterial pressure (MAP) (A) in women without and with a hypertensive disorder during pregnancy (non-HDP: circle, grey and HDP: square, black; respectively). The percentage prescription of angiotensin converting enzyme inhibitor (ACEi) and angiotensin receptor blocker (ARB) (B) and anti-hypertensive medication (anti-HTN) (C) in women without and with a hypertensive disorder during pregnancy (non-HDP: white bar and HDP: black bar; respectively). *p<0.05 vs non-HDP at the respective visit. P for interaction: $p_{group*visit}$ HDPs vs non-HDPs (GEE-analysis corrected for age and BMI).*

Case-control study – characteristics

Baseline characteristics of the women included in the case-control sub-study at time point 0 (visit 1 PREVEND study) and pregnancy data corresponding to the pregnancy used for the analysis (index pregnancy) are shown in Table 2. In total, 97 women had not had their index pregnancy at time point 0 (Control n=74, PIH n=14, PE/HELLP n=9). BMI, MAP, antihypertensive use, gestational age at delivery, and birth weight in the PIH and PE/HELLP group were different as compared to the Control group. At baseline, eGFR and 24-albuminuria were not significantly different between the groups. Ten years postpartum, blood pressure was significantly higher in the PIH and PE/HELLP group as compared to Control (Table 2 and Figure 3A).

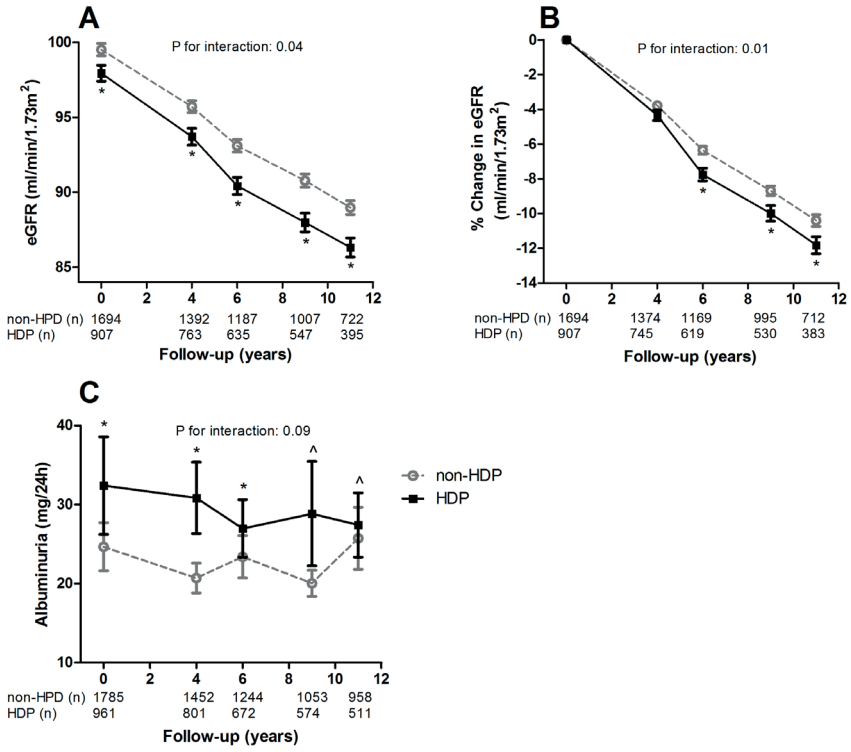


Figure 2. Prospective data on renal function and albuminuria in women with and without HDP.

*The estimate marginal means (EMM) ± standard error (SE) of the estimated glomerular filtration rate (eGFR) (A), the mean ± SD of the percentage decline in eGFR at every visit compared to visit 1 (B), and the EMM ± SE of the 24-hour albuminuria levels (C) in women without and with a hypertensive disorder during pregnancy (non-HDP: circle, grey and HDP: square, black; respectively). *p<0.05, ^p<0.1 vs non-HDP at the respective visit. P for interaction: $p_{group*visit}$ HDPs vs non-HDPs (GEE-analysis corrected for age and BMI).*

Case-control study – renal outcomes

Figure 3 also shows renal outcomes of the case-control study 10 years postpartum. eGFR was significantly lower in PE/HELLP group compared to Control group 10 years postpartum (99.5±29.9 vs 108.0±9.1; p=0.01; Figure 3B). No significant difference was found between PIH and Control group (105.2±14.5 vs 108.0±9.1; p=0.29). 24-hour albuminuria was significantly higher in PE/HELLP as compared to Control group (10.7 (6.3-21.1) vs 7.0 (5.7-10.3); p=0.03; Figure 3C). Although numbers are small, 4.1% of the Control group, 7.9% of the PIH group, and 17.4% of the PE/HELLP group showed 24-albuminuria levels >30mg/24-hour (Chi-square: p=0.049, Linear-by-Linear Association: p=0.02). 10 years postpartum, 7.8% of the Control group, 10.9% of the PIH group, and 19.2% from the PE/HELLP group suffered from CKD (Chi-square: p=0.18, Linear-by-linear association: p=0.08; Figure 3D). The OR and 95% CI for CKD risk with controls as reference group was 1.76 (0.93-3.34; p=0.08).

Validity questionnaire

Sensitivity was 69%; from a total of 72 hypertensive women, i.e. a history of a HDP according to their medical record, 50 women self-reported an HDP in the questionnaire. Specificity was 91%; from a total of 150 Control women, 136 women without a history of HDP according to their medical record, self-reported not to have had an HDP.

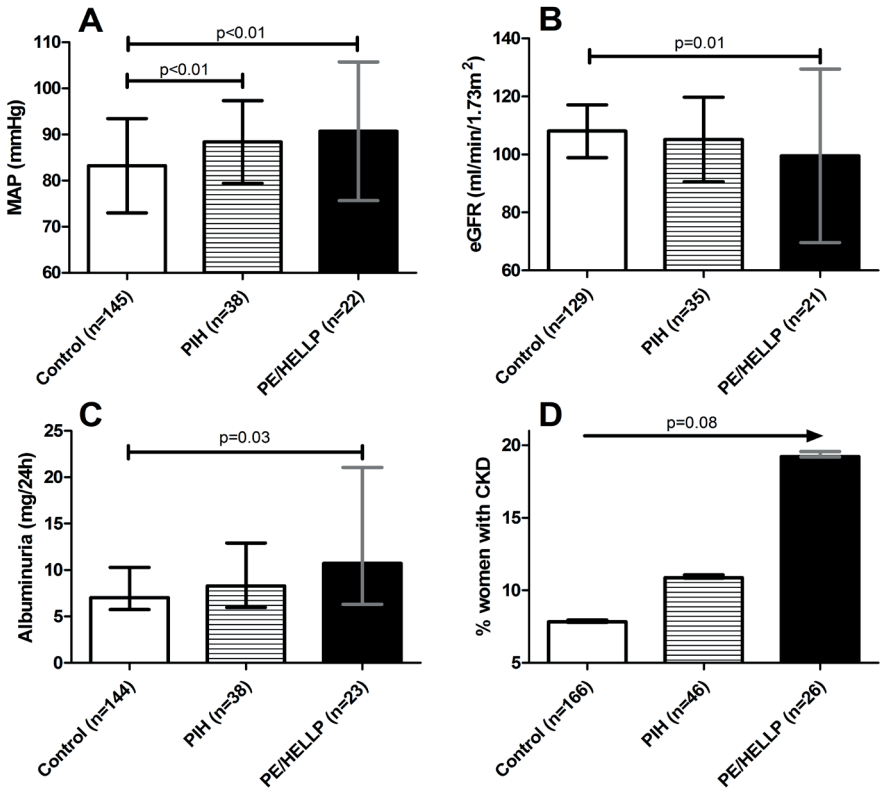


Figure 3. Blood pressure and renal function 10-year post-partum in the case-control.

The mean ± SD of the mean arterial pressure (MAP) (A) and the estimated glomerular filtration rate (eGFR) (B) and the median (25^{th}-75^{th} percentile) of 24-hour albuminuria (C) and the percentage (95% CI) of women suffering from chronic kidney disease (CKD) (D) 10 years postpartum in control (white bar), in pregnancy induced hypertension (PIH; striped bar) and preeclampsia or the HELLP-syndrome group (PE/HELLP; black bar). (D) CKD was defined as as a combination of an eGFR <60ml/min per 1.73m² and/or 24-hour albuminuria >30mg, the p-value of the odds ratio is presented.

Table 2. Characteristics of the case-control study

	Control (N=202)	PIH (N=56)	PE/HELLP (N=29)
Baseline[1]			
Maternal characteristics			
Age (years)	34.8 ± 3.9	35.6 ± 4.4	35.4 ± 4.2
Caucasian (n (%))	187 (92.6)	53 (94.6)	27 (93.1)
BMI (kg/m²)	23.6 ± 3.4	26.4 ± 4.6*	25.4 ± 5.8*
SBP (mmHg)	111 ± 9	122 ± 15*	118 ± 12*
DBP (mmHg)	65 ± 6	72 ± 8*	71 ± 6*
Job (n (%))	139 (69)	35 (63)	16 (55)
Current smoker (n (%))	65 (32)	21 (38)	12 (41)
Alcohol use (n (%))	34 (17)	8 (14)	4 (14)
Antihypertensive use (n (%))	2 (1)	5 (9)*	4 (15)*
ACE-i and ARB use (n(%))	0 (0)	0 (0)	0 (0)
Oral contraceptive use (n(%))	90 (45)	17 (33)	12 (41)
Cardiovascular comorbidity (n ((%))	0 (0)	1 (2)	0 (0)
Medical history (n (%))			
Asthma/COPD	15 (7)	4 (7)	1 (3)
Thyroid disorders	7 (4)	2 (4)	1 (3)
Arthritis	5 (3)	1 (2)	1 (3)
Epilepsy	0 (0)	0 (0)	0 (0)
Laboratory results			
Glucose (mmol/l)	4.3 (4.0 - 4.6)	4.4 (4.1-4.9)	4.4 (4.2 - 4.9)
Insulin (mmol/l)	6.7 (5.2 – 9.8)	7.7 (6.1 - 10.6)	8.3 (5.6 - 11.7)
HOMA	1.3 (1.0 - 1.9)	1.5 (1.2 - 2.2)*	1.6 (1.1 - 2.5)

	Control (N=202)	PIH (N=56)	PE/HELLP (N=29)
Cholesterol (mmol/l)	4.9 (4.3 - 5.4)	5.1 (4.3 - 5.9)	5.3 (4.5 - 6.0)*
Uric acid (mmol/l)	0.24 (0.22 - 0.27)	0.26 (0.21 - 0.29)	0.26 (0.22 - 0.29)
Triglycerides (mmol/l)	0.89 (0.69 - 1.2)	1.06 (0.73 - 1.37)	1.02 (0.77 - 1.38)
Renal function			
Creatinine (mmol/L)	63 ± 9	62 ± 11	67 ± 31
Cystatin C (mg/L)	0.77 ± 0.1	0.79 ± 0.1	0.83 ± 0.3*
eGFR (mL/min/1.73m^2)	113 ± 11	111 ± 13	110 ± 19
Albuminuria (mg/24h)	7.4 (5.7-11.1)	9.1 (6.3-14.5)	9.6 (5.9-18.5)
ESRD (n (%))	0 (0)	0 (0)	0 (0)
Pregnancy data[2]			
Highest DBP during pregnancy (mmHg)	74 ± 6	95 ± 7*	101 ± 10*,†
Age at delivery (years)	33.1 ± 4.1	32.6 ± 5.1	33.1 ± 4.9
Gestational age at delivery (week^{+days})	40^{+3} (39^{+2}-41^{+2})	39^{+6} (38^{+4}-40^{+6})*	38^{+3} (36^{+5}-40^{+1})*
Birth weight child (grams)	3554 ± 461	3256 ± 716*	2833 ± 873*,†
Gestational diabetes (n (%))	35 (17)	8 (14)	8 (28)
10 years postpartum			
Age (years)	43.2 ± 4.5	41.6 ± 4.7	43.9 ± 4.9
BMI	24.5 ± 3.7	26.5 ± 4.4*	25.6 ± 6.4
SBP (mmHg)	114 ± 14	119 ± 14*	125 ± 22*
DBP (mmHg)	68 ± 9	73 ± 7*	74 ± 12*

*Data are presented as mean ± SD or median (25^{th} – 75^{th} percentile) unless otherwise stated. [1]Data from visit 1 of the PREVEND study; [2]Data corresponding to the complicated pregnancy included in analysis. PIH: pregnancy induced hypertension; PE: preeclampsia; HELLP: haemolysis elevated liver enzymes and low platelets syndrome; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; ACE-i: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; COPD: chronic obstructive pulmonary disease; HOMA: homeostatic model assessment index; eGFR: estimated glomerular filtration rate; ESRD: end stage renal disease. *p<0.05 vs Control, †p<0.05 vs PIH.*

DISCUSSION

We are the first to show longitudinal data on renal function in relation to hypertensive disorders during pregnancy. We found both a lower eGFR and a steeper eGFR decline over time in women with a history of a self-reported hypertensive disorder of pregnancy, even after correction for ACE-i and ARB use. Our case-control study with details on severity of the hypertensive disorder during pregnancy demonstrated a graded effect on renal function in relation to the severity of the HDP, with the lowest eGFR and highest albuminuria in the PE/HELLP group 10 years postpartum. Moreover, as compared to controls, a higher percentage of women with a history of PE/HELLP met the criteria for CKD. The lower postpartum eGFR and accelerated decline in eGFR during follow-up in the HDP group fits with the observation that these women are at increased risk for ESRD [3-5].

To our knowledge, only one other study investigated renal function decline after an HDP. In this study, Spaan et al. suggested that renal function decline with age was comparable between formerly preeclamptic women and healthy controls [16]. However, their study was cross-sectional in design assessing a different cohort of women of 20 years older, and groups were relatively small. Several other studies showed either subtle or no abnormalities in renal function in women with a history of an HDP at set time points postpartum. Significantly lower para-aminohippurate measured effective renal plasma flow was found in formerly preeclamptic women compared to matched controls [17]. A meta-analysis revealed no significant differences in creatinine clearance and eGFR between formerly preeclamptic women and healthy controls [7]. A recent report showed a high-normal eGFR in women with a history of early-onset preeclampsia, 10 years postpartum [8]. A high-normal GFR might indicate an early stage of hyperfiltration, which is in line with the slightly increased filtration fraction observed in formerly early-onset preeclamptic women, in the absence of co-morbidity [9]. An increased filtration fraction, generally considered an unfavourable renal hemodynamic profile, might provide a mechanistic explanation for loss of GFR during long-term exposure [18]. Our current finding of a lower eGFR and a steeper eGFR decline after HDP in a large population with repeated measurements during long term follow-up therefore adds robust and valuable insights on the long-term course of renal function after hypertensive pregnancy.

Data on the development of CKD after an HDP are sparse. Our date clearly suggest that women with a history of an HDP have a higher risk for developing CKD at middle age. This is in line with large population-based studies investigating the association between a history of an HDP and the risk of ESRD [3-5] and a small study focussing on the association between preeclampsia and kidney diseases by assessing kidney biopsies [19]. Our results of a steeper decline in eGFR plus the increased risk for CKD in women with a history of an HDP, suggest an acceleration of the age-related renal function decline and more frequent occurrence of CKD.

This paper is also the first presenting long-term longitudinal albuminuria data postpartum. Within our cohort, higher albuminuria levels were found in the HDP group, however, this difference trended to resolve 6 years after entry of the study. Resolution of albuminuria might be due to the increased ACE-i and ARB use during follow-up in the HDP as compared to the non-HDP group. Sampling error in 24-hour urine samples and lost to follow-up could as well been of influence on

these data. In addition, a significantly higher albuminuria level 10 years postpartum in women with a history of PE/HELLP as compared to controls was found, irrespective of ACE-i and ARB use. This is in line with a meta-analysis reporting an occurrence of micro-albuminuria of 31% in formerly preeclamptic women [7]. Recent studies not included in the meta-analysis reported lower incidences of albuminuria in formerly preeclamptic women, varying from no difference [6, 20] to only 1.1% 10 years postpartum [8].

Postpartum development of hypertension might mediate the association between the hypertensive disorder during pregnancy and increased risk for ESRD later in life [5]. A recent meta-analysis showed a relative risk of 3.13 for hypertension in formerly preeclamptic women [21]. In our study, mean arterial pressure was on average 8 mmHg higher in the HDP group. This is in accord with previous large studies [22, 23]. Both severity (SBP >160mmHg or DBP >110mmHg) and early-onset PE (<34 weeks) were shown to increase the risk of cardiovascular diseases even more [24]. We show the same graded increase, with the highest blood pressure in the PE/HELLP group 10 years postpartum. Our longitudinal data do not support a more pronounced increase in blood pressure during the complete follow-up in HDP compared to non-HDP women. This might be influenced by blood pressure lowering treatment, which showed a significant increase in use over time in the HDP group. Whether the increased blood pressure found in the HDP group contributes to the decline in renal function during follow-up remains therefore unknown.

The exact mechanisms underlying the association between HDP and ESRD need to be elucidated. On one hand pre-pregnancy common risk factors for an HDP and renal impairments might explain these findings [25, 26], while on the other hand an HDP itself might induce renal impairment [27-29], or a combination of both. Unfortunately, we did not have sufficient cases with both pre-pregnancy and post-pregnancy data to analyse this important research question in detail.

Limitations of our studies are the retrospective character based on self-reported HDP. In addition, since the mean age of women included in the case-control study was lower than the mean age of the women in the PREVEND cohort, the women studied in the case-control study are not representative for the large PREVEND population. More non-HDP were using OCC, which is likely due differences in prescription behaviour due to comorbidity. Earlier analysis of the PREVEND study showed that OCC use has negative influence on eGFR and albuminuria [30]. Since our HDP group, with the steeper eGFR decline had less OCC use, this cannot explain our results found. Of note, another large study showed no effect on eGFR when analysing post-menopausal use of estrogen [31].

Hypertensive disorders in pregnancy complicate around 10% of the pregnancies. However, in our cohort, 35% of the women reported a history of an HDP. We considered two main factors that might explain this finding. At first, our population was enriched with subjects with microalbuminuria, meaning that our population studied is at risk for CVD and CKD, and therefore true incidence for HDP is likely to be higher. Our microalbuminuria enriched population might also clarify our higher number of women reporting dialysis at baseline as compared to the true incidence in the Dutch population (Renine database, the Netherlands). Secondly, over reporting of an HDP could have been present, since women visiting for antenatal care might interpret the physiological rise in blood pressure towards the end of pregnancy as a hypertensive disorder, as well as influences of recall bias.

To validate the maternally self-reported history of an HDP, we conducted a specificity and sensitivity analysis by combining our PREVEND cohort with the case-control, showing a specificity 91% and a sensitivity of 69%. Our sensitivity analysis also suggests under reporting of an HDP, meaning that our non-HDP group includes true HDP women, potentially leading to a reduction of the differences found. Accuracy of our data are in accordance with earlier studies [32], but the self-reported nature of pregnancy complications clearly is a limitation of our data.

In conclusion, women with a history of an HDP have a slightly but significantly lower eGFR and steeper long-term decline in eGFR postpartum as compared to women without a history of an HDP on long-term follow-up. In addition, we found that women with a history of PE/HELLP have a higher risk to develop CKD on average 10-years postpartum. The increased renal function decline might contribute to the increased risk for ESRD later in life that has been reported after HDP. Based on our high-risk population, regular follow-up of women with a history of severe HDP (PE/HELLP) is of importance to take advantage of the window of opportunities for preventive treatment. Future studies should explore this risk in the general population in order to obtain more accurate estimates of the renal risk after HDP.

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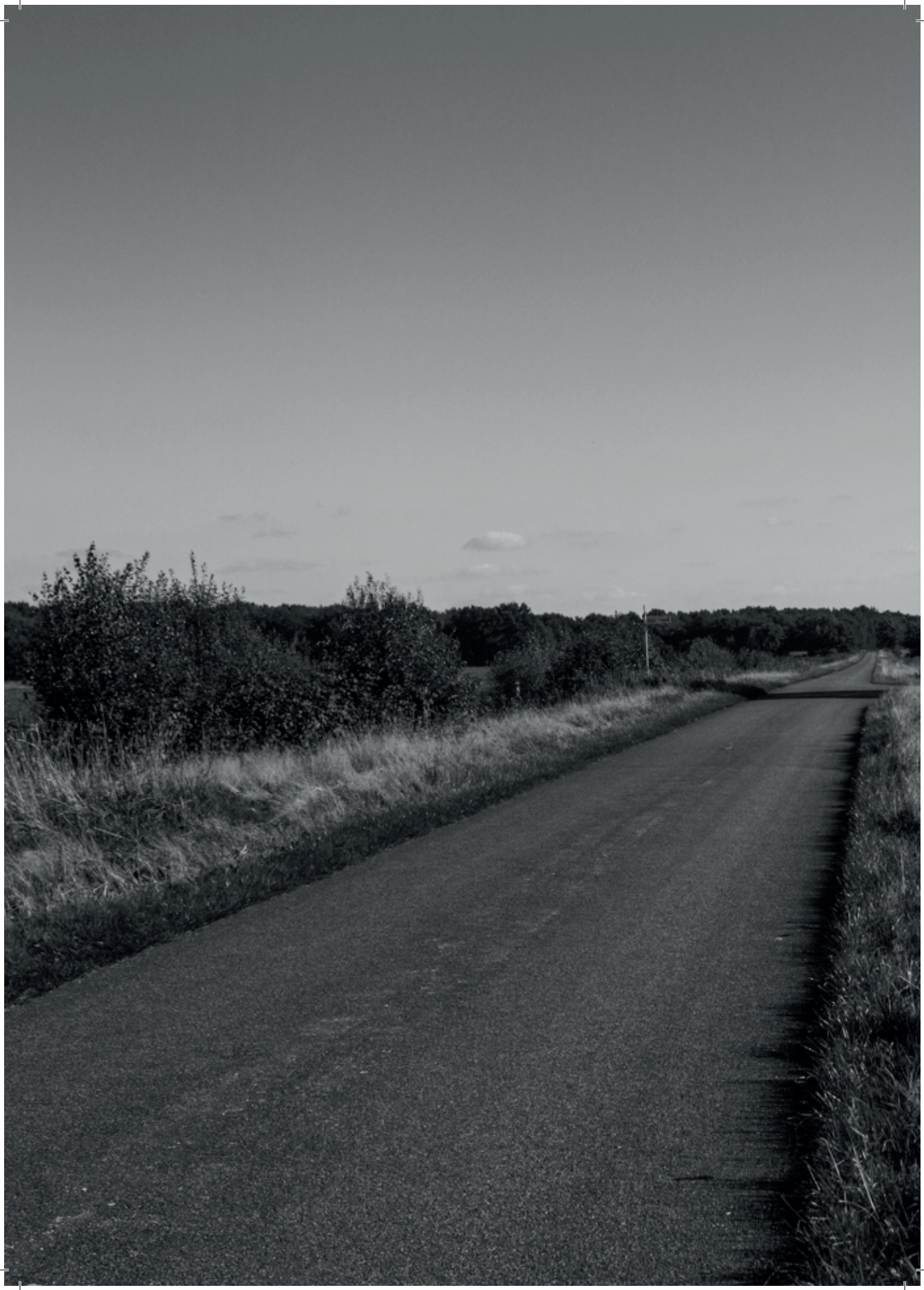

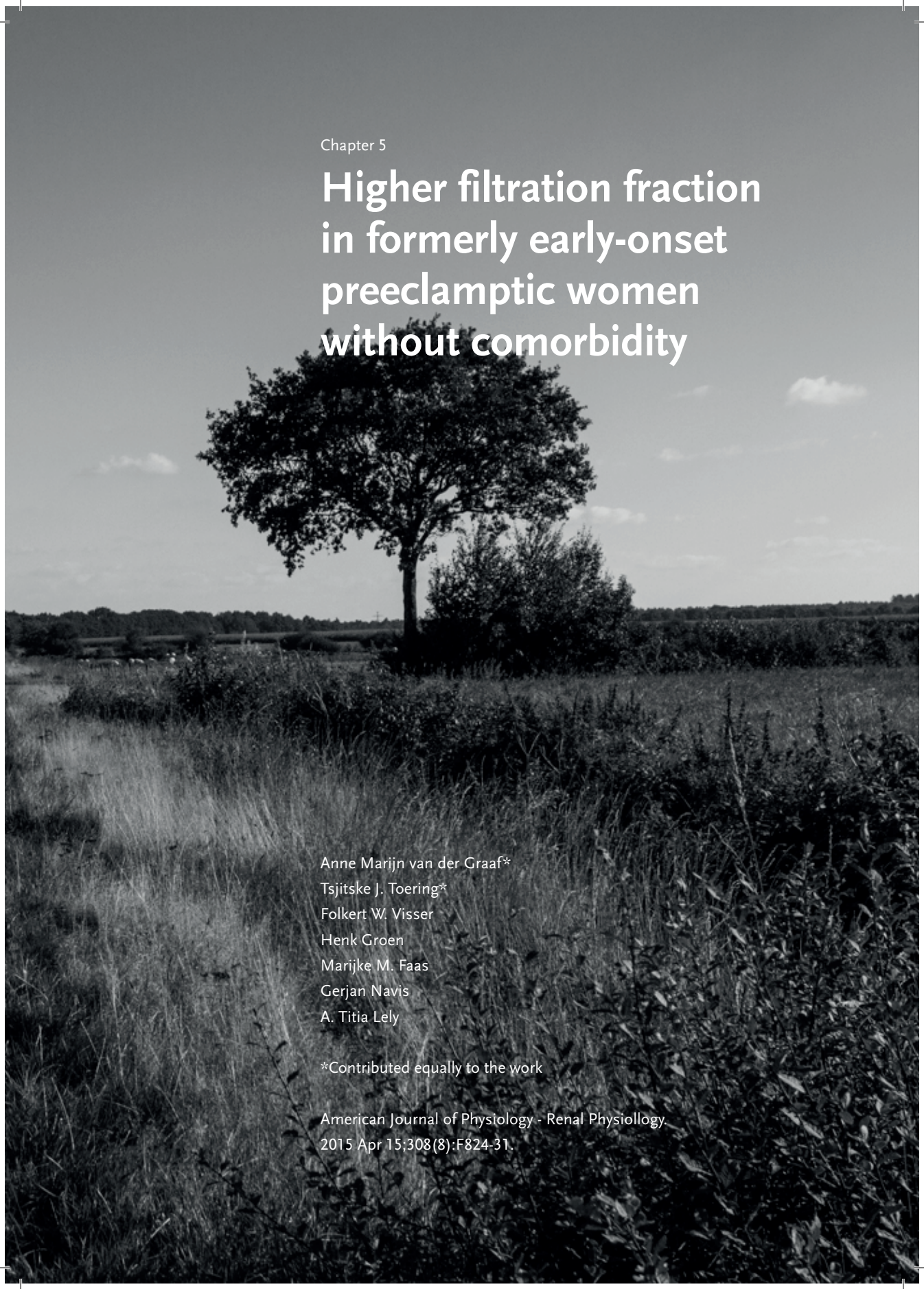

Chapter 5

Higher filtration fraction in formerly early-onset preeclamptic women without comorbidity

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ABSTRACT

Formerly preeclamptic women have an increased risk for developing end stage renal disease, that has been attributed to altered renal hemodynamics and abnormalities in the renin-angiotensin aldosterone system. Whether this is due to preeclampsia itself or to co-morbid conditions is unknown. Renal hemodynamics and responsiveness to angiotensin II during low sodium (7 days 50 mmol Na^+/24h) and high sodium intake (HS; 7 days 200 mmol Na^+/24h) were studied in 18 healthy normotensive formerly early-onset preeclamptic women (fPE-women) and 18 healthy controls (fHP-women), all selected for absence of co-morbidity. At the end of each diet, renal hemodynamics and blood pressure were measured before and during graded angiotensin II infusion. Both HS intake and former preeclampsia increased filtration fraction (FF) without an interaction between the two. FF was highest during HS in fPE-women (0.31 ± 0.12 vs fHP-women: 0.29 ± 0.11, GEE analysis BMI corrected, p=0.03). Renal response to angiotensin II infusion was not different between groups. In conclusion, fPE-women have a higher FF compared to fHP-women. As this was observed in the absence of co-morbidity, preeclampsia itself might exert long-term effects on renal hemodynamics. However, we cannot exclude the presence of pre-pregnancy alterations in renal function which in itself lead to an increased risk for preeclampsia. In experimental studies, an elevated FF has been shown to play a pathogenic role in the development of hypertension and renal damage. Future studies, however, should evaluate whether the subtle differences in renal hemodynamics after preeclampsia contribute to the increased long-term renal risk after preeclampsia.

INTRODUCTION

Complicating up to 8% of pregnancies, preeclampsia (PE) is a major cause of maternal and fetal morbidity and mortality worldwide [27]. PE is characterized by de-novo development of hypertension and proteinuria during the second half of pregnancy. Although it is a pregnancy-specific disease, evidence has mounted that PE has important long-term implications for maternal health, in particular cardiovascular and renal health [2, 24, 33]. It is, however, uncertain whether the increased renal and cardiovascular risk in formerly preeclamptic women is explained by PE itself, or by underlying common (pre-pregnant) risk factors and co-morbidity.

Recent data showed that formerly preeclamptic women have a five to fourteen fold higher risk for developing end stage renal disease (ESRD) [33, 36]. Moreover, women who experienced multiple preeclamptic pregnancies have an even higher risk for ESRD [33]. The risk for developing cardiovascular disease (CVD) is especially high for women who have a history of early-onset PE (before 34 weeks of gestational age) [2]. It is unknown whether this also applies for the risk of developing ESRD. However, in a large Norwegian cohort study the association between former PE and developing ESRD is stronger in formerly preeclamptic women who had given birth to preterm infant or child with low birth weight. Early-onset preeclamptic women often give birth to a preterm infant or child with low birth weight. Therefore, this suggests that these women might have a higher risk for developing ESRD [33]. The exact mechanisms underlying the increased risk for CVD and ESRD in formerly preeclamptic women are not completely understood [30].

There are data, albeit sparse, showing that formerly preeclamptic women have persistent abnormalities in renal hemodynamics early and late after pregnancy, as a possible early pathway of increased renal risk [25, 29]. However, this was mainly found in hypertensive women and thus might relate to the hypertension per se, rather than to the former PE specifically. Moreover, it is important to note that the renal hemodynamic profile is closely interlinked with sodium homeostasis and the renin-angiotensin aldosterone system (RAAS) [31]. Sodium intake modulates renal hemodynamics in healthy subjects [34] as well as in subjects with hypertension [19]. In risk populations like sodium-sensitive hypertensive and overweight subjects, high dietary sodium intake elicits an unfavorable renal hemodynamic profile, which is absent during low sodium diet [8, 13]. With regard to blood pressure response, both sodium sensitivity and altered response to angiotensin II (ang II) is reported in formerly preeclamptic women [12, 23, 37]. The role of sodium intake in renal hemodynamics and the renal response to ang II in relation to sodium intake in formerly preeclamptic women is still unknown.

Therefore, in the present study, we investigated the renal hemodynamic profile in women with a history of early-onset PE, compared with healthy controls during both low and high sodium intake. In order to preclude the effect of differences in co-morbidity between the groups, we carefully selected healthy normotensive Caucasian formerly preeclamptic women, without co-morbidity, with a body mass index (BMI) < 30 kg/m^2 and excluded hypertensive formerly preeclamptic women.

In steady state during low and high sodium intake, glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and filtration fraction (FF) were measured at baseline and during graded ang II infusion. We hypothesized that previous PE would be associated with changes in the renal hemodynamic profile, be it or not dependent on sodium intake, as candidate mechanism for the increased risk for long-term renal damage in formerly preeclamptic women.

METHODS

Study population

We identified 264 formerly early-onset preeclamptic women (referred to as formerly preeclamptic women) one to ten years after delivery from an electronic delivery database of the department of Obstetrics and Gynecology at the University Medical Center Groningen (UMCG). Medical records were reviewed for accuracy of diagnosis of PE. PE was defined according to the International Society for the Study of Hypertension in Pregnancy criteria [6]. Early-onset PE was defined as developing PE before 34 weeks of gestational age. A total of 224 formerly early-onset preeclamptic women were invited by mail to participate in the study. Twenty-four of these women were willing to participate and were invited for a screening visit to the UMCG. After the screening visit, one women was excluded for using antihypertensive medication, one woman because of high blood pressure during the screening visit, one woman was using hormonal suppletion which could not temporarily stopped and three woman were excluded for other reasons (pregnancy, time consuming, post-menopausal). Each of the 18 remaining formerly preeclamptic women was matched for age and year of index pregnancy (within one year) with a parous control (referred to as control group) whose pregnancy had been uncomplicated and normotensive. These women from the control group were recruited either through the department's electronic delivery database or by advertisement. Their records were evaluated to confirm that pregnancy was indeed uneventful.

All women were non-smokers and normotensive, having a sitting systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg measured at screening by an automatic sphygmomanometer (Dinamap®, G.E. Medical Systems, Milwaukee, Winsconsin, USA) and were not treated with an antihypertensive drug. Blood pressure was measured at screening at both arms to check for presence of a clinical significant difference in blood pressure (present in none of the subjects). Physical examination and electrocardiography did not reveal any abnormalities. None of the women had (a history of) underlying renal disease or hypertension, nor were they obese (i.e. BMI<30 kg/m^2 at screening). They also did not have diabetes or a history of gestational diabetes, nor were they currently pregnant or lactating or using oral contraception. The study was approved by the local medical ethical committee (METc-number: 2010/294) and all women gave written informed consent in accordance with the Declaration of Helsinki. The study was registered in the Netherlands National Trial Register (www.trialregister.nl; trial registration number: 2635) as REsponse To Angiotensin II in formerly Preeclamptic women (RETAP) study.

Study protocol

This cross-over protocol consisted of two one-week periods with at least four weeks in between, a 7-day period on low sodium diet (LS; aim: 50 mmol Na^+/day) and a 7-day period on a high sodium diet (HS; aim: 200 mmol Na^+/day). For assessment of dietary compliance and the achievement of a stable sodium balance 24-hour urine was collected at day 3 and day 6 during each period. During the last day of the dietary week, blood pressure was measured during a period of 24-hours by ambulatory blood pressure measurement (ABPM; Spacelabs Healthcare). The cuff was placed around the non-dominant arm at the brachial level. The recorders were programmed to measure blood pressure at a 20-min interval during daytime and at an hourly interval during nighttime (10pm till 6am). Women were asked to fill out a diary during this 24-hour to differentiate between day- and nighttime measurements and to correct for intense exercise afterwards. The nocturnal fall in blood pressure (dipping) was defined as the percentage decline in nocturnal blood pressure as compared to daytime values. In our study, non-dippers were defined as individuals with less than 10% decline in nocturnal blood pressure as compared to daytime blood pressure.

Both renal hemodynamics and ang II responsiveness are greatly influenced by sex hormones [21]. To avoid influence of these hormones, all measurements were performed during the mid-follicular phase (day 7±2 of menstrual cycle). At day 7 of both study periods, the subjects reported at the research unit at 8am after an overnight fast. Body weight, length and waist-to-hip ratio were measured at the start of this day. An intravenous cannula was inserted into each forearm, one for drawing blood samples, the other for infusion of radio-labeled tracers and ang II. Subjects received standardized meals and fluids during the day, with sodium intake adjusted to the prescribed diet. To ensure sufficient urine output, infusion of 250 mL/h of 5% glucose was administered and every hour 250 mL of oral fluids were provided.

GFR and ERPF were measured from the clearance of constantly infused radio-labeled tracers, ^{125}I-iothalamate (IOT) and ^{131}I-Hippuran (HIPP), respectively, in semi-supine position in a quiet room as described before [1, 35]. After drawing a time point-0 blood sample, a priming solution containing 20 mL infusion solution (0.04 MBq of IOT and 0.03 MBq of HIPP) plus an extra amount of 0.6 MBq of IOT was given at 08.00 h, followed by a constant infusion of 12 mL/h. Plasma concentrations of both tracers are allowed to stabilize during 1.5-hour equilibration, which is followed by two 2-hour periods for simultaneous clearances of IOT and HIPP. The latter are calculated as (U*V)/PIOT and (I*V)/PHIPP, respectively. U*V represents urinary excretion of the tracer; I*V, the infusion rate of the tracer, which equals clearance from plasma during steady state; P, tracer values in plasma at the end of each clearance period. The plasma clearance (I*V)/PHIPP equals its urinary clearance because there is no extrarenal clearance of this tracer. Thus, when plasma levels are in steady state, ERPF equals I*V/PHIPP. GFR is calculated as the urinary clearance of IOT, corrected for voiding errors: (U*V/P)corr. As urinary clearance of HIPP equals plasma clearance in case of perfect urine collection, we routinely use the ratio of plasma-to-urinary clearance of HIPP to correct urinary clearance of IOT for voiding errors and dead space. By this method, coefficient of variation for GFR is 2.5% and for ERPF 5%. FF was calculated by the ratio of GFR and ERPF. Extra cellular volume (ECV) was estimated from the distribution volume of IOT and is calculated from

the plasma level of IOT in the body, which equals the amount infused minus the amount excreted. It is calculated as ((I*V+B*V)-(U*V))/P. B*V represent the bolus infusion of the tracers [35]. GFR, ERPF and ECV were indexed for body surface area (BSA), by dividing the raw sample by BSA and multiplying it with 1.73m^2. BSA was calculated according to the DuBois-DuBois formula [10].

Blood pressure and heart rate were measured by using an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA) at 15-min intervals, with subjects being in a quiet room, in a semi-supine position, with their arm in resting position. Appropriate blood pressure cuff was determined on the basis of arm circumference. Mean arterial pressure (MAP) was calculated as diastolic pressure plus one-third of the pulse pressure. Renal blood flow (RBF) was calculated as ERPF/(1–hematocrit). Renal vascular resistance (RVR) was calculated as MAP/RBF × 80,000. Baseline values for blood pressure and GFR and ERPF were obtained from 10am to 12pm. Between 12pm and 3pm ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously, at a constant rate in doses of 0.3, 1 and 3 ng/kg/min each during 1-hour. During these ang II infusions blood pressure was measured at 5-min intervals.

Sampling and chemical analysis of urine and blood samples

Fasting blood samples were drawn for analysis of hematocrit (Ht), glucose, HbA1C, sodium, potassium, creatinine, and thyroid stimulating hormone (TSH). Measurements were performed by the use of an automated clinical chemistry analyzer (Sysmex hematology analyzer (for Ht), Sysmex Tosoh G8 (for HbA1c) and Roche Modular). Fasting serum insulin was determined by an automated immunoassay analyzer (Architect, Abbott). Homeostasis model assessment (HOMA) was calculated by: glucose (mL/L) x insulin (microunits/mL)/22.5. Blood for measuring plasma aldosterone and plasma renin activity (PRA) was collected at 11am in precooled tubes and immediately centrifuged at 4°C for 10min (3000 rpm). Plasma was subsequently stored in -80°C until analysis. Aldosterone was measured with a commercially available radioimmunoassay kit (coat a count RIA, Siemens). PRA was determined by a radioimmunoassay kit that detects the production of angiotensin I due to the enzymatic activity of plasma renin acting on endogenous plasma angiotensinogen (nanograms of angiotensin I produced per liter of plasma per hour; RIA, CisBio International, France). Urine samples were drawn from the 24-hour urine collected by all women. The level of urinary sodium, potassium, creatinine, and albumin were assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel). As some study subjects were still slightly menstruating during the 24-hour urine collections, these samples were not suitable for albuminuria measurement. Therefore, to test for albuminuria, a random morning urine sample of all subjects was collected after the end of the study, at a point in time where subjects were certain not to menstruate to exclude confounding by admixture of blood.

Statistical analysis

Data were analyzed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA) and GraphPad prism 5.01 (GraphPad Software Inc. San Diego, CA, USA). Parametric data are presented as mean ± standard deviation (SD) in text, tables and figures. Non-parametric data are expressed as median with 25th and 75th percentile. Differences in baseline characteristics, blood and urinary parameters between formerly preeclamptic women and controls were tested with the Student t-test for parametric data and Mann-Whitney U test for non-parametric data. For 24-hour blood pressure data, dipping was analyzed with the Chi-square test. For MAP and renal hemodynamics, to separately test the effects of sodium intake (factor diet), history of PE or not (factor group), and the interaction between the two (factor diet*group), a generalized estimating equations (GEE) analysis was performed. In the GEE analysis we corrected FF and MAP for difference in BMI. This repeated measurements analysis is appropriate for this small cross-over study with repeated measures in one subject [16]. In comparisons a p-value <0.05 was considered statistically significant.

Power calculation

The cross-over design of the study with multiple factors resulted in a multivariate power calculation. The main endpoint of the RETAP study was renal response (GFR, ERPF and FF) to ang II after low and high sodium diet in formerly preeclamptic subjects compared to healthy controls. In the multivariate power calculation 3 factors (response to ang II, low and high sodium diet and control group vs. formerly preeclamptic women) and 2 confounders were taken into account. Therefore, 25 women per group (n=10*5/2) were needed to perform multivariate analysis. Due to the low incidence of early-onset PE and the demanding nature of the study, we were not able to include 25 women per group in our hospital. However, after including 18 women per group and performing an interim analysis, we found a significant difference between both groups.

RESULTS

Baseline characteristics

Baseline characteristics of the two groups are shown in Table 1. There were no statistically significant differences in age, number of pregnancies (gravidity), number of births (parity) and time since last pregnancy between the two groups. 15 out of 18 women experienced the early-onset PE during their first pregnancy, the other three women experienced PE during their second pregnancy. Formerly preeclamptic women had a higher weight and consequently a higher BMI compared with the control group. Both groups showed an increase of approximately 1.5 kilogram in weight during high sodium intake compared to low sodium intake (p<0.001). Waist-to-hip ratio was not significantly different between the two groups.

The average 24-hour blood pressure values are shown in Table 1 and were similar in formerly preeclamptic women and control subjects. Both groups responded to high sodium intake reflected by an increased blood pressure. However, the average salt-induced increase in blood pressure did not differ between the two groups. Data are presented for 24-hour averages but similar

results are present when analyzing diurnal and nocturnal values separately. The number of women showing the nocturnal fall of MAP (dipping pattern) was not significantly different between the two groups.

Table 1. Baseline characteristics

Characteristic	History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	P
Age, years	36 ± 5	36 ± 5	.951
Gravidity	2.5 ± 1.3	2.6 ± 1.1	.951
Parity	2.0 ± 0.7	2.2 ± 1.0	.589
Time since last pregnancy, years	4.2 ± 2.6	5.3 ± 3.0	.243
Weight LS, kg	66.1 ± 8.3	73.2 ± 10.5	**.029**
Weight HS, kg	67.9 ± 8.3*	74.9 ± 11.0*	**.039**
BMI LS, kg/m^2	22.6 ± 2.6	25.3 ± 3.3	**.010**
BMI HS, kg/m^2	23.2 ± 2.7	25.9 ± 3.5	**.015**
Waist-to-hip ratio	0.83 ± 0.04	0.84 ± 0.06	.443
24-h MAP LS, mmHg	87 ± 5	88 ± 8	.89
24-h MAP HS, mmHg	90 ± 7*	90 ± 8*	.71
Dipping MAP LS no/yes (%yes)	1/17 (94 %)	3/13 (81%)	.23
Dipping MAP HS no/yes (%yes)	3/15 (83%)	5/11 (69%)	.32

*Data are presented as mean ± SD. BMI, body mass index; MAP, mean arterial blood pressure; LS, low sodium; HS, high sodium. *p<0.05 vs low sodium within the group.*

Blood and urinary parameters

Blood and urinary parameters are shown in Table 2. No statistically significant differences in hematocrit, fasting glucose, insulin, HOMA, HbA1C, plasma sodium, plasma potassium, plasma creatinine, and TSH were found between the two groups. In both groups, plasma creatinine was significantly lower during high sodium intake compared with low sodium intake (p=0.001).

No statistically significant differences in urinary sodium excretion, potassium excretion and urea excretion were found between the groups; this reflects an equal intake of sodium, potassium and proteins between the two groups. Formerly preeclamptic women had a higher urinary creatinine excretion compared with control subjects.

No differences in PRA and aldosterone were found between the groups. In both groups, a significant decrease in PRA and aldosterone was found during high sodium intake compared to low sodium intake ($p<0.001$), which reflects that in both groups the systemic RAAS is adequately and similarly modulated by sodium intake.

There were no differences in urinary albumin/creatinine ratio between the groups.

Table 2. Blood and urinary parameters

Parameter	History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	P
Hematocrit LS, l/l	0.40 ± 0.02	0.40 ± 0.03	.460
Hematocrit HS, l/l	0.38 ± 0.03	0.38 ± 0.03	.634
Glucose LS, mmol/L	5.1 ± 0.7	5.0 ± 0.5	.628
Glucose HS, mmol/L	5.0 ± 0.5	5.0 ± 0.3	.605
Insulin LS, µU/mL	8.35 (5.60-9.90)	8.50 (6.50-12.80)	.542
Insulin HS, µU/mL	7.10 (4.70-9.30)	7.65 (4.60-10.80)	.525
HOMA LS	1.91 (1.26-2.16)	1.83 (1.33-3.01)	.636
HOMA HS	1.55 (0.97-2.15)	1.69 (1.08-2.30)	.369
HbA1c LS, mmol/mol	35 (31.75-36.00)	33 (32.50-35.00)	.134
HbA1c HS, mmol/mol	35 (32.75-37.25)	34 (30.75-35.25)	.203
Plasma sodium LS, mmol/L	140 ± 1.6	140 ± 1.9	.853
Plasma sodium HS, mmol/L	142 ± 1.8	141 ± 2.4	.164
Plasma potassium LS, mmol/L	4.0 ± 0.2	3.9 ± 0.3	.604
Plasma potassium HS, mmol/L	3.9 ± 0.2	3.9 ± 0.2	.287
Plasma creatinine LS, µmol/L	66.5 ± 9.2	70.1 ± 9.0	.242
Plasma creatinine HS, µmol/L	61.1 ± 7.0*	65.7 ± 9.3*	.090
Plasma TSH LS, mU/L	1.29 ± 0.6	1.60 ± 0.9	.245
Plasma TSH HS, mU/L	1.48 ± 0.5	1.47 ± 0.9	.975
Urinary sodium LS, mmol/24 h	38.9 ± 14.0	45.1 ± 22.8	.326

Parameter	History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	P
Urinary sodium HS, mmol/24 h	220.8 ± 63.5*	258.4 ± 85.9*	.145
Urinary potassium LS, mmol/24 h	66.2 ± 21.3	76.3 ± 25.2	.202
Urinary potassium HS, mmol/24 h	79.8 ± 33.5	73.3 ± 14.7	.459
Urinary creatinin LS, mmol/24h	9.8 ± 1.9	11.1 ± 1.0	**.013**
Urinary creatinin HS, mmol/24h	9.8 ± 1.9	11.5 ± 2.4	**.013**
Urinary urea LS, mmol/24h	264 ± 91	306 ± 63	.119
Urinary urea HS, mmol/24h	339 ± 89*	340 ± 65	.973
PRA LS, nmol Ang-I/L/h	0.80 (0.50-1.20)	0.85 (0.70-1.50)	.501
PRA HS, nmol Ang-I/L/h	0.20 (0.10-0.50)*	0.20 (0.09-0.30)*	.584
Aldosterone LS, pmol/L	255 (204-395)	341 (214-477)	.161
Aldosterone HS, pmol/L	71 (29-93)*	59 (35-96)*	.839
Urinary albumin/creatinine, g/mol	0.6 ± 0.3	0.5 ± 0.4	.212

*Data are expressed as mean ± SD or as median (25^{th}-75^{th} percentile). LS, low sodium; HS, high sodium; HOMA, homeostatic model assessment index (HOMA was calculated as [glucose*insulin/22.5]); TSH, thyroid stimulating hormone; PRA, plasma renin activity; Ang-I, angiotensin I. *p<0.05 vs low sodium within the group.*

Blood pressure and renal function at baseline

Blood pressure and renal function during high and low sodium intake are shown in Table 3 and Table 4, and Figure 1. By performing GEE analysis, we found no differences in MAP between both groups (p_{group}=0.401). High sodium intake significantly increased MAP in both groups to a similar extent (p_{diet}<0.001; $p_{diet*group}$=0.414, no interaction between diet and group).

With regards to renal hemodynamics, no differences were found in GFR (p_{group}=0.688) and ECV (p_{group}=0.973) between both groups. Formerly preeclamptic women have a slightly lower ERPF compared to control subjects, but this did not reach statistical significance (p_{group}=0.253). However, FF was significantly higher in formerly preeclamptic women compared to healthy controls (p_{group}=0.035). High sodium intake significantly increased GFR, FF and ECV in both groups to a similar extent (GFR: p_{diet}<0.001, $p_{diet*group}$=0.824; FF: p_{diet}=0.025, $p_{diet*group}$=0.460; ECV: p_{diet}<0.001, $p_{diet*group}$=0.766). However, there was no effect of sodium intake on ERPF (p_{diet}=0.127, $p_{diet*group}$=0.683). The higher FF in formerly preeclamptic women was not explained by MAP; no significant correlation could be detected between MAP and FF (r=0.095; p=0.581).

Blood pressure and renal function during ang II infusion

Graded ang II infusion showed in both groups a dose-dependent rise in blood pressure during both high and low sodium intake (Table 4). No significant differences were found in absolute blood pressure values during ang II infusion between both groups. Figure 2 demonstrates ERPF during ang II infusion. Both groups showed a dose-dependent decrease in ERPF during ang II infusion (p_{dose}<0.001). No differences were found in the responses of ERPF to ang II between the groups (p_{group}=0.337). Sodium intake did not affect the response of ERPF to ang II infusion (p_{diet}=0.562).

Table 3. Baseline renal function parameters

Parameter	History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	P
RVR LS, dyne*s/cm^5	10985 ± 2936	11026 ± 2017	.962
RVR HS, dyne*s/cm^5	11248 ± 3071	11461 ± 2388	.819
ERPF LS, mL/min/1.73m^2	625 ± 146	622 ± 99	.949
ERPF HS, mL/min/1.73m^2	642 ± 149	621 ± 105	.623
Creatinine clearance LS, mL/min	107 ± 28	111 ± 17	.591
Creatinine clearance HS, mL/min	114 ± 24*	124 ± 25*	.220
eGFR LS, mL/min/1.73m^2	102 ± 14	97 ± 15	.368
eGFR HS, mL/min/1.73m^2	109 ± 10*	103 ± 15*	.117

*Data are presented as mean ± SD. LS, low sodium; HS, high sodium; RVR, renal vascular resistance; ERPF, effetive renal plasma flow; eGFR, estimated glomerular filtration rate (eGFR was calculated by using CKD-epi formula [15]). *p<0.05 vs low sodium within the group.*

Table 4. Blood pressure at baseline and during angiotensin II infusion

MAP during ang II infusion	History of normotensive pregnancy (n=18)	History of preeclamptic pregnancy (n=18)	P
Low sodium			
Baseline, mmHg	81 ± 7	83 ± 8	.375
0.3 ng/kg/min, mmHg	81 ± 8	82 ± 8	.745
1.0 ng/kg/min, mmHg	86 ± 9	87 ± 9	.705
3.0 ng/kg/min, mmHg	92 ± 12	95 ± 10	.477
Recovery, mmHg	85 ± 8	87 ± 10	.612
High sodium			
Baseline, mmHg	85 ± 8	86 ± 9	.714
0.3 ng/kg/min, mmHg	84 ± 9	85 ± 10	.601
1.0 ng/kg/min, mmHg	89 ± 9	93 ± 11	.260
3.0 ng/kg/min, mmHg	99 ± 8	100 ± 10	.646
Recovery, mmHg	89 ± 8	89 ± 11	.981

Data are presented as mean ± SD. MAP, mean arterial pressure; ang II, angiotensin II

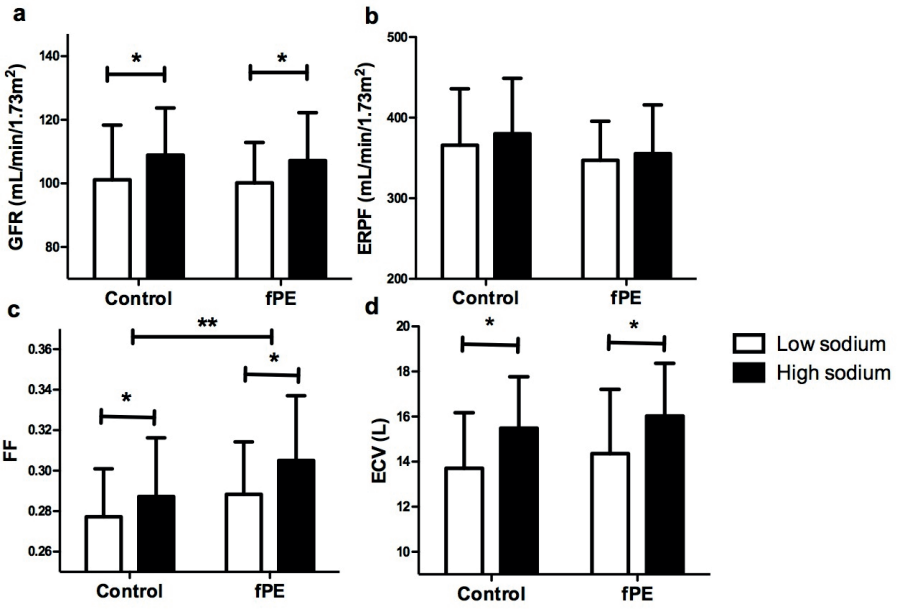

Figure 1. Renal hemodynamics during low and high sodium diet in control women and in formerly preeclamptic women.

*Glomerular filtration rate (GFR) (A), effective renal plasma flow (ERPF) (B), filtration fraction (FF) (C) and extra cellular volume (ECV) (D) at baseline during low sodium (white bars) and high sodium (black bars) diet in women with history of normotensive pregnancy (control) and in formerly preeclamptic women (fPE). Data are expressed as mean ± SEM * p<0.05 low vs high sodium intake, **p<0.05 control vs fPE (GEE analysis; FF is corrected for BMI). No interaction between dietary sodium response and group.*

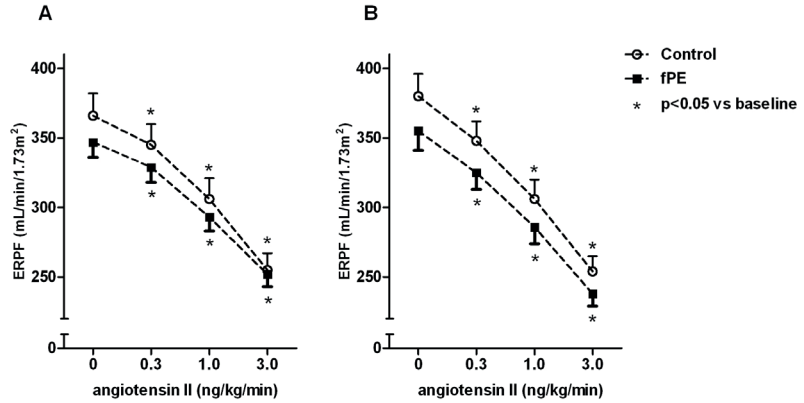

Figure 2. Effective renal plasma flow in response to angiotensin II.

Effective renal plasma flow (ERPF) during angiotensin II infusion during low sodium (A) and high sodium (B) in formerly preeclamptic women (fPE) and women with history of normotensive pregnancy (control). No significant differences were found between the groups (GEE-analysis).

DISCUSSION

This is the first study investigating renal hemodynamics in healthy formerly early-onset preeclamptic women compared with women with a history of a normotensive pregnancy, during a low and high sodium diet with graded ang II infusion. Although blood pressure was not different, a slightly, but significantly higher FF was present in formerly early-onset preeclamptic women on either sodium intake. There was no difference in renal response to ang II infusion during either low or high sodium intake. Thus, healthy women with a history of early-onset PE, in the absence of co-morbidity, have slight but persistent subtle differences in renal hemodynamics compared to matched controls, irrespective sodium intake. Whether differences in renal hemodynamics resulted from the previous PE, or were already present pre-pregnancy, cannot be derived from our data.

Our study is the first to report altered renal hemodynamics in healthy formerly preeclamptic women in the absence of co-morbidity. Indeed, 24-hour blood pressure and blood pressure response to sodium intake were all in the normal range and comparable between the two groups. Prior studies in formerly preeclamptic women described more pronounced changes in renal hemodynamic profile, but in these studies co-morbidity, namely hypertension, might well explain the renal hemodynamic findings of a higher FF and RVR with lower ERPF [25, 29]. A recent study in women 5-10 years after severe early-onset PE showed marked increased sodium sensitivity [17]. In line, previous studies with mixed phenotypes and co-morbidity, reported increased blood pressure responsiveness to ang II postpartum [12, 23, 26].

The hypothesis that PE per se may predispose to altered renal function and kidney damage is in line with other findings. Vikse et al. reported that familial aggregation of risk factors does not explain the increased ESRD risk after PE [32]. Furthermore, Chambers et al. demonstrated impaired endothelial function in women with a history of PE, independent of established risk factors [9]. Yet, these data do not exclude the possibility of pre-pregnancy changes in renal hemodynamics that predispose to both PE and later renal damage. Animal studies could provide a clean model of PE to elucidate whether ESRD and cardiovascular damage after PE are causally related to PE itself. In for example an experimental sFlt-1 mice model, levels of proteins involved in several vascular diseases were increased postpartum [7].

Microalbuminuria is thought to persist in a substantial amount of women after PE, reported in a meta-analysis with a mixed patient population (including diabetes mellitus) [18]. However, in a recent large Norwegian study, PE was not associated with microalbuminuria [22]. In line with the results from Sandvik et al, a morning urine sample collected after completion of our study did not show a difference in albuminuria between the groups; all women had albuminuria values within the normal range.

A higher FF, even within the normal range, can be considered a candidate mechanism for development of hypertension and renal damage, proposed by Brenner et al. [5], mainly based on micropuncture studies in rats. Based on these studies, an increased FF is assumed a proxy for elevated glomerular capillary pressure, thus contributing to progressive renal damage during long-term exposure [11]. Data on the pathogenic role of elevated FF in human are scarce, but we previously

showed that a mildly elevated FF is independently associated with worse long term renal outcome and mortality in renal transplant recipients [3].

The mechanism underlying the higher FF in the current study cannot be established with certainty, as this would require micropuncture. Hemodynamic as well as structural differences of the glomerular microvasculature are possible. The nominally lower ERPF in the formerly preeclamptic women is compatible with a shift towards more efferent vasoconstriction relative to afferent vasotonus. Several neurohumoral factors could elicit such a pattern, alone or in combination, including the sympathetic nervous system, vasopressin, natriuretic peptides and/or other factors, and increased activity of the RAAS [11]. A reduction of increased intraglomerular pressure by the use of RAAS blockade in healthy subjects reinforces the role of the RAAS in impaired renal hemodynamics, i.e. hyperfiltration [20]. However, we found no differences in circulating parameters of RAAS-activity or in ang II renal responsiveness. A higher BMI, even in the non-obese [4], and central fat distribution [14] have also been associated with hyperfiltration, but in our study body fat distribution was similar between the groups, and the slight difference in BMI could not explain the difference in FF, as tested by multivariate analysis. Alternatively, a redistribution of renal blood flow towards juxtacortical glomeruli, with a higher FF, could be present. Differences in sodium and protein intake are not likely to have elicited the difference in FF since urinary sodium and urea levels, reflecting intake of sodium and protein, were comparable between the groups. Finally, structural microvascular differences could be involved, although the reduction in FF during sodium restriction demonstrates that there is at least a partial hemodynamic component. So far, it is thought that glomerular changes (glomerular endotheliosis, accompanied by decreased GFR) during PE resolve completely after PE [28].

High sodium intake induced an increase in FF in both groups. This renal hemodynamic response is in line with studies in sodium-sensitive hypertensive individuals and overweight subjects, where sodium elicited hyperfiltration [8, 13]. We did not find an interaction between the effect of sodium intake and previous PE on FF. However, considering the aligned role of increased FF in long-term renal risk, our data suggest that sodium restriction, and/or RAAS-blockade, could exert a beneficial effect on long-term renal risk. Obviously long-term studies are required to substantiate such an assumption.

Our study has several limitations. Firstly, due to our strict inclusion and exclusion criteria our sample size is relatively small. In addition, the range in years post-partum is relatively broad although not different between the two groups. Furthermore, our data do not provide a mechanism for the differences in renal hemodynamics, as we found no differences in RAAS-parameters, we did not assess parameters of the sympathetic nervous system, and diet was not standardized for protein intake. Finally, our data cannot distinguish between an effect of previous PE on renal hemodynamics, or, alternatively, altered renal hemodynamics pre-pregnancy.

In conclusion, formerly early-onset preeclamptic women, have a slightly, but significantly higher FF than controls, in the absence of co-morbidity. Theoretically, a slightly higher FF contributes to the increased renal risk that has been reported after PE, but this assumption requires substantiation by future long-term studies. Furthermore, prospective studies, preferably starting pre-pregnancy, should elucidate whether the higher FF is induced by the previous PE, or was already present pre-pregnancy.

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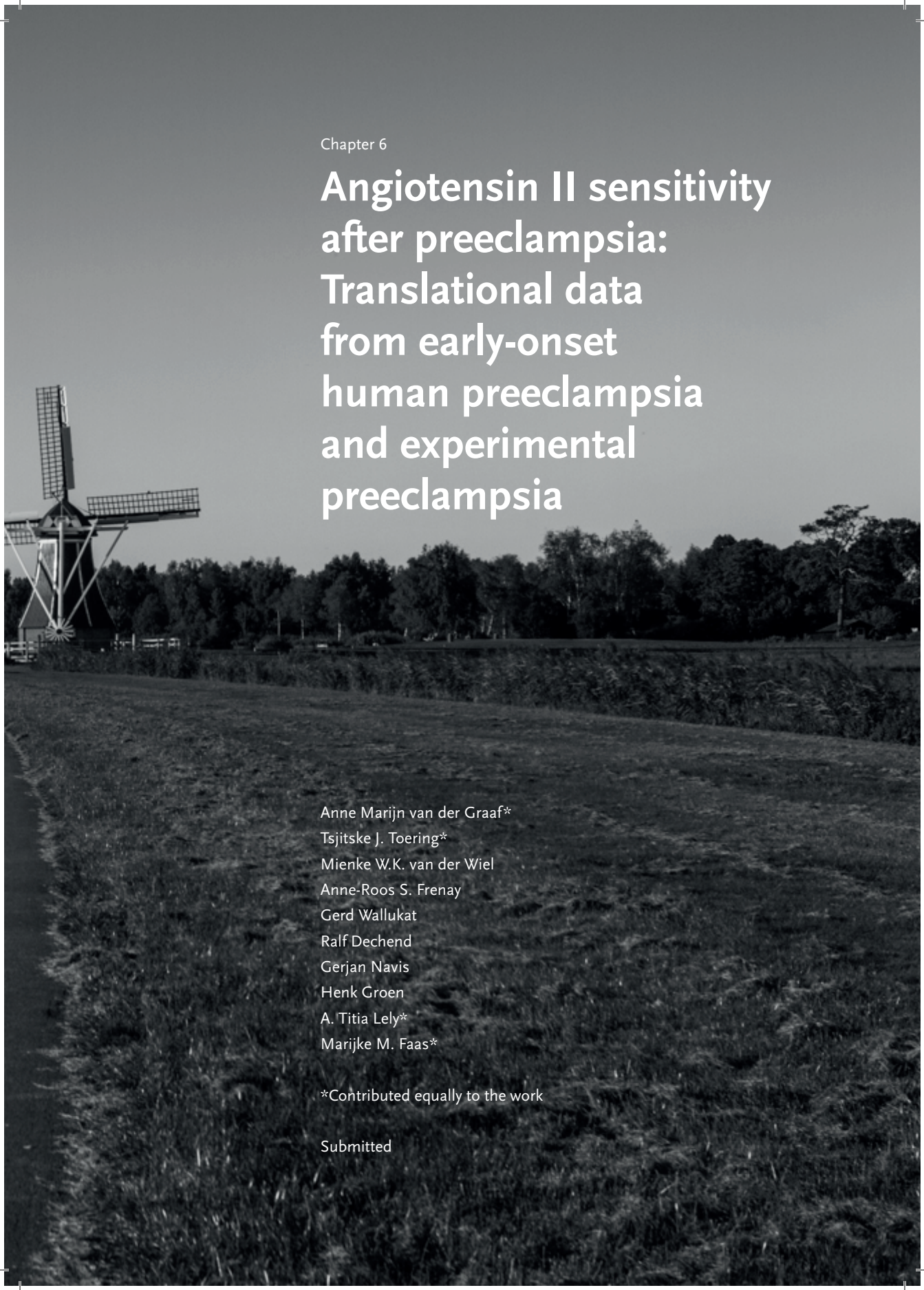

Chapter 6

Angiotensin II sensitivity after preeclampsia: Translational data from early-onset human preeclampsia and experimental preeclampsia

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ABSTRACT

Formerly preeclamptic women have an increased risk for cardiovascular and renal diseases later in life. Whether this is due to preeclampsia itself or co-morbid conditions is unknown. Our aim was to establish, in a translational approach, whether the increased angiotensin II sensitivity in preeclampsia persists postpartum and whether preeclampsia itself plays a role. In 18 formerly healthy early-onset preeclamptic and 18 formerly healthy pregnant women (controls) baseline blood pressure and responses to angiotensin II (0.3, 1.0, and 3.0ng/kg/min) were measured. Simultaneously, in never-pregnant, formerly healthy pregnant, and formerly experimental preeclamptic rats, angiotensin II sensitivity was studied; in-vivo by measuring changes in blood pressure and proteinuria during angiotensin II infusion with osmotic minipumps (200ng/kg/min), and *ex-vivo* by vascular reactivity measurements. In humans, no difference in baseline mean arterial blood pressure was seen (85 ± 8 vs 86 ± 9; p=0.71), while 1.0ng/kg/min angiotensin II showed a trend towards an increased response in formerly preeclamptic women vs controls (p=0.057). In rats, chronic infusion of angiotensin II showed a trend towards a larger rise in systolic blood pressure (69.4% vs 53.8%; p=0.068) and a significantly higher systolic blood pressure at termination in formerly preeclamptic vs never-pregnant rats (159.5 ± 29.5 vs 136.7 ± 16.8; p=0.046). In response to angiotensin II, there was a significant increase in proteinuria in formerly preeclamptic rats only. In conclusion, healthy formerly early-onset preeclamptic women and formerly experimental preeclamptic rats exhibit subtle persistent increased angiotensin II sensitivity. Together, these data support a role for preeclampsia itself in altered angiotensin II sensitivity postpartum.

INTRODUCTION

Preeclampsia is a pregnancy specific syndrome, clinically characterized by the presence of hypertension and proteinuria in the second half of pregnancy [1]. It is a leading cause of maternal and perinatal mortality. Women that suffered from preeclampsia, especially early-onset preeclampsia, have an increased risk for cardiovascular- and renal diseases later on in life [2-5].

The common hypothesis is that pre-existing vascular and metabolic risk factors cause both preeclampsia and later cardiovascular and renal disease [6]. Data, however, from the Hunt-cohort showed that only approximately 50% of the increased risk after preeclampsia could be explained by common risk factors [7]. Indeed, it has been demonstrated that preeclampsia itself could lead to kidney and endothelial damage since in a large cohort familial aggregation did not explain the increased risk for end-stage renal disease (ESRD) [8]. These studies suggest that preeclampsia itself may also contribute to the increased risk to develop cardiovascular and renal damage after preeclampsia. However, studies above are epidemiological studies, which are not optimal to answer the question whether preeclampsia itself contributes to the increased vascular risk.

Mechanistic studies in formerly preeclamptic women have shown that the increased angiotensin II (ang II) sensitivity, which is present during preeclampsia [9], persisted after preeclampsia; albeit more subtle [10, 11]. However, the formerly preeclamptic women in these studies had multiple comorbidities, such as hypertension and obesity, which makes it difficult to discriminate between the effect of common risk factors and the effect of preeclampsia itself. Furthermore, sodium intake, menstrual cycle period and time-after pregnancy were not completely standardized in these studies and may have been of influence.

We hypothesized that preeclampsia itself may play a role in the persistent increased ang II sensitivity. Therefore, we investigated blood pressure response upon ang II infusion in healthy, normotensive, formerly early-onset preeclamptic women, selected for absence of comorbidity and without signs of underlying diseases. Furthermore, we performed a rat study; to test the hypothesis that preeclampsia itself may induce persistent increased ang II sensitivity. We used healthy rats in which we induced preeclampsia by infusion of low dose lipopolysaccharide (LPS) [12] during pregnancy, and measured blood pressure and renal responses to chronic ang II infusion as well as *ex-vivo* ang II sensitivity in the aorta postpartum.

MATERIALS AND METHODS

Human study

Study population

The study was approved by the local medical ethical committee (METc-number: 2010/294) and all women gave written informed consent in accordance with the Declaration of Helsinki. The study was registered in the Netherlands National Trial Register (www.trialregister.nl; trial register number: 2635) as REsponse To Angiotensin II in formerly Preeclamptic women (RETAP) study.

Thirty-six healthy, normotensive postpartum Caucasian women were studied at the University Medical Center Groningen (UMCG); 18 healthy women with a history of early-onset preeclampsia (fPE-women) and 18 healthy women with a history of uncomplicated, normotensive pregnancy (fHP-women) were used as a control group. 264 women with a history of early-onset preeclampsia one to ten years ago were selected from an electronic delivery database of the UMCG Obstetrics department. Medical records were reviewed for accuracy of diagnosis of preeclampsia, which was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy [13]. Early-onset preeclampsia was defined as developing preeclampsia before 34 weeks of gestation. Women with a history of renal disease, using any antihypertensive medication, with a BMI>30 kg/m^2 at screening, with diabetes or a history of gestational diabetes were excluded. Also current pregnancy, current lactation, being post-menopausal, and use of oral contraception [14, 15] were used as exclusion criteria.

224 early-onset fPE-women were invited by mail to participate in the study. In total, 24 of these women were willing to participate and were invited for a screening visit to the UMCG. After the screening visit, one woman was excluded for using antihypertensive medication and one woman was using hormonal suppletion which could not be temporarily stopped, one woman was excluded because of hypertension measured during the screening visit, and 3 women were excluded for other reasons (pregnancy, time-consuming protocol, post-menopausal). Each of the remaining fPE-women was matched for age and year of index pregnancy (within one year) with a parous control whose pregnancy had been uncomplicated and normotensive. These fHP-women were recruited either through the department's electronic delivery database or recruited amongst hospital/department employees and their family members. Their records were evaluated to confirm that their pregnancy was indeed uneventful. Exclusion criteria as described above for the cases were applied. All subjects were non-smokers and normotensive at screening, having a sitting systolic blood pressure (SBP) <140mmHg and diastolic blood pressure (DBP) <90mmHg measured by Dinamap (the average of three measurements was taken). Physical examination and electrocardiography did not reveal any abnormalities.

Study protocol

In the week prior to the measurements, women were asked to use a sodium standardized diet (aim: 200 mmol Na^+/24-hour urine) starting from day one of their menstrual cycle. To assess dietary compliance, 24-hour urine was collected at day 3 and day 6 of the dietary week and results were discussed with all women. During the last day of the dietary week, blood pressure was measured during a period of 24-hours by ambulatory blood pressure measurement (ABPM; Spacelabs Healthcare). The cuff was placed around the non-dominant arm at the brachial level. The recorders were programmed to measure blood pressure at a 20-min interval during daytime and at an hourly interval during nighttime (10pm till 6am). Women were asked to fill out a diary during this 24-hour to differentiate between day- and nighttime measurements and to correct for intense exercise afterwards.

At the study day, women reported at the research unit at 8.00am after an overnight fast. Body weight was measured at the start of this day. An intravenous cannula was inserted into each forearm, one for drawing blood samples, the other for infusion of ang II. During the measurements, women were sitting in semi-supine position in a quiet room. All women received standardized meals and fluids during the day. Blood pressure and heart rate were measured by the use of an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA) at 15-min intervals. Mean arterial pressure (MAP) was calculated as 2-times DBP plus SBP divided by 3. Baseline values for blood pressure were obtained from 10.00am to 12.00pm. Between 12.00pm and 3.00pm ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously, at a constant rate in doses of 0.3, 1.0, and 3.0 ng/kg/min each during 1-hour. During the ang II infusions blood pressure was measured at 5-min intervals.

Blood and urine sampling and analysis

Fasting blood samples were drawn for analysis of hematocrit (Ht), glucose, glycated hemoglobin (HbA1C), insulin, and creatinine. Measurements were performed by the use of an automated clinical chemistry analyzer (Roche Modular; Sysmex heamatology analyzer (for Ht) and Sysmex Tosoh G8 (for HbA1C)). Fasting serum insulin was determined by an automated immunoassay analyzer (Architect, Abbott). Homeostasis model assessment (HOMA) was calculated by: (glucose x insulin)/22.5. Blood samples for baseline assessments (non-fasting samples) were drawn at 10.00am. These plasma samples were collected in pre-cooled tubes and immediately centrifuged at 4°C, 956g for 10 min and subsequently stored at -80°C until analysis of aldosterone and plasma renin activity (PRA). Aldosterone was measured with a commercially available radioimmunoassay kit (coat a count RIA, Siemens). PRA was measured with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess endogenous angiotensinogen (nanograms of angiotensin I produced per liter of plasma per hour; CisBio International, France). To analyze auto-antibodiess against the angiotensin II type I receptor (AT1-AA) IgG fractions for the neonatal cardiac contraction assay were prepared and isolation and cultivation of the neonatal heart cells were performed as described previously [16]. Using the chronotropic responses to AT1-R mediated stimulation of cultured neonatal rat cardiomyocytes coupled with receptor-specific antagonists the AT1-AA were detected [17].

Urine samples were drawn from the 24-hour urine collected at day 6 of the dietary week by all women. The levels of sodium, creatinine and albumin were assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel). The creatinine clearance was then calculated using the formula: (urinary creatinine/24h*1000)/plasma creatinine. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-epi formula [18]. This creatinine-based equation has been shown to accurately estimate the GFR in a healthy population. As some study subjects were still slightly menstruating during the 24-hour urine collections, these samples were not suitable for albuminuria measurement. Therefore, to test for albuminuria, a random morning urine sample was collected after completion of the study, at a point in time where subjects were certain not to menstruate to exclude confounding by admixture of blood.

Power analysis

This study was originally designed to assess both blood pressure and renal response to ang II after low and high sodium diet in formerly preeclamptic women compared to healthy controls. The cross-over design of the study with several main end-points resulted in a multivariate power calculation. In the multivariate power calculation 3 factors (response to ang II, low and high sodium diet and control group vs. formerly preeclamptic women) and 2 confounders were taken into account. We calculated the total number of participants by n=10*5 (3 factors and 2 confounders). Therefore, 25 subjects per group were needed. Due to the low incidence of early-onset preeclampsia and the intensiveness of the study protocol, we were not able to include 25 women per group in our hospital. However, after including 18 women per group and performing an interim analysis, we found a significant difference on our renal function end point between both groups (not shown in this paper). Therefore, we decided to stop including at n=18 after an interim-analysis. Since we describe our secondary endpoint in this paper, as an explorative study, no separate power analysis was performed.

Data analysis

Statistical analysis was performed using SPSS for Windows (Version 20.0) on a standard computer. Parametric data are presented as mean ± standard deviation (SD) in text, table and figures, unless otherwise stated and analyzed using Student t-test. Nonparametric data are presented as median (25^{th}-75^{th} percentile) and analyzed using Mann-Whitney U. Chi-square was used to analyze the AT1-AA and Pearson Correlation to test the correlation of AT1-AA with time since index pregnancy. Univariate linear regression was performed to test whether BMI was of influence on the differences found. Blood pressure responses upon the different ang II infusion steps was analyzed using the general estimating equations (GEE-analyses; mixed model analysis). Baseline blood pressure, BMI and an interaction term (group (fHP and fPE) * ang II infusion step) were entered as covariates, and exchangeable was chosen as correlation matrix. GEE-analyses was also performed to test whether the presence of AT1-AA and "time since index pregnancy" was of influence on the ang II sensitivity. In all cases, differences were considered significant if $p<0.05$.

Animal study

Animals

Experiments were conducted under protocols approved by the Animal Ethical Committee of the University of Groningen. Wistar rats (Harlan Inc, Horst, the Netherlands) were kept in a 12-hour light-dark cycle and constant room temperature, with food and water freely available in the home cages. Until selection for experiments vaginal smears were taken daily. Rats were rendered pregnant by housing them on pro-oestrus with fertile males for one night. Day 0 of pregnancy was documented by the presence of spermatozoa in the vaginal smear. Subsequently, pregnant rats were randomly allocated into two groups; healthy pregnant rats (HP-rats, n=19) or experimental preeclamptic rats (PE-rats, n=22). As controls, rats that have never been pregnant were used (NP-rats, n=25).

All rats were equipped with a permanent cannula inserted into the right jugular vein under isoflurane/oxygen anesthesia on day 0 of pregnancy, according to standard methods [19]. The permanent jugular vein cannula allows stress free infusion of lipopolysacharide (LPS;E-Coli, 0.55: B5, Whittaker MA Bioproducts, Walkerville, Md.) or saline.

Experimental set up

At day 14 of pregnancy, rats received a 1-hour low dose LPS infusion or a 1-hour saline infusion. Non-pregnant rats received saline infusion 2 weeks after cannulation. To study the long-term effects of experimental preeclampsia the following experimental set-up was chosen. Six weeks postpartum and nine weeks after cannulation in NP-rats, baseline values for blood pressure and proteinuria were assessed. Then, in each group of rats, 50% of the rats received ang II infusion continuously via an osmotic minipump, whereas the other 50% received a sham pump for three weeks. Blood pressure, proteinuria and creatinine clearance (only after 3 weeks) were measured weekly during this infusion period in all rats. Subsequently, after three weeks of infusion rats were sacrificed and kidneys and aortas were collected. Kidneys were checked for kidney inflammation (macrophages influx) and kidney damage (deposition of αSMA). To evaluate whether the in-vivo effect of ang II was due to increased vascular responsiveness to ang II, the aortas of the rats with sham pumps were used for aortic contraction experiments.

Induction of experimental preeclampsia

On day 14 of pregnancy pregnant rats randomized for the experimental preeclampsia group were infused with LPS (1µg/kg bw in 2 ml saline in 1-hour) according to standard methods [12]; rats allocated to the healthy pregnant group were infused with only saline (2 ml in 1-hour). Non-pregnant rats received saline infusion according to the same protocol. After delivery, pups were immediately dissociated from their mothers to avoid an effect of lactation. The number of alive and dead pups was counted and weight and length of the pups that were alive, i.e. moving at time of measurements, were measured. Two weeks after delivery, the permanent jugular vein cannulas were surgically removed by a small incision under isoflurane/oxygen anesthesia.

In-vivo ang II infusion and sacrifice of the rats

Six weeks after delivery, the NP-rats, the fHP-rats, and the fPE-rats were further randomly divided in a control group (c-NP, n=12, c-fHP, n=10, and c-fPE, n=11) receiving sham pumps intraperitoneally (ip) for 3 weeks and a group receiving an osmotic minipump with ang II ip (infusion rate: 200ng/min/kg in saline containing 0.01 N acetic acid; A-NP, n=13; A-fHP, n=9; and A-fPE, n=11; Alzet, Cupertino, CA, model 2004) for 3 weeks. Blood pressure and proteinuria were measured weekly. Three weeks after pump implantation, rats were anesthetized and aortic blood pressure (see below) was measured before termination by heart puncture. After termination, the left kidney was harvested and parts of the kidney were placed in 4% paraformaldehyde in PBS or snap frozen. The thoracic aorta was also harvested. Small parts of the thoracic aorta were fixed in 4% paraformaldehyde or snap frozen. Since we were interested in the effect of former preeclampsia on the *ex-vivo* vascular

reactivity, without confounding by prior chronic ang II infusion, we used the thoracic aortas of the rats treated with sham pumps for vascular reactivity experiments.

Measurement of blood pressure

Blood pressure was measured at baseline (i.e. 6 weeks after delivery) and weekly for 3 weeks during sham or ang II infusion using an indirect tail-cuff plethysmographic method with a rat tail blood pressure monitor (Apollo 179; IITC Life Science, Woodland Hills, California, USA). All rats were conscious during the measurements. In order to reduce spontaneous variation in blood pressure, rats were extensively trained for a period of four weeks on a daily basis. Prior to blood pressure readings, rats were optimally warmed using a warmth lamp to induce vasodilation of the tail vein. Readings were repeated ten times and after excluding the lowest value the average of the lowest three remaining values for SBP was used for further analysis. In addition, blood pressure was measured at termination (i.e. after 21-days of infusion) using a bed-side monitor (Datex-Ohmeda, Cardiocap/5). Rats were anesthetized with isoflurane/oxygen and the abdominal cavity was opened (100% O_2, 0.8mL/min, 5% isoflurane for induction followed by 2%). A catheter was inserted in the abdominal aorta and the blood pressure was noted after 20 seconds of recording.

Blood and urine sampling and analysis

Rats from all groups were placed in metabolic cages for 24-hour urine collection prior to implantation of the osmotic minipumps, to assess baseline proteinuria. After pump implantation, proteinuria was measured weekly for 3 weeks. Urinary concentrations of protein (Pyrogallol Red – Molybdate Complex) were determined as previously described [20] and 24-hour excretion rates were calculated. To determine creatinine, a blood sample was collected in a pre-cooled tube at termination (day 21) and immediately centrifuged at 4°C, 956g for 10min and plasma was subsequently stored at -80°C until analysis. Urinary and plasma creatinine concentrations were determined (CREA plus, cobas, Roche Modular, Basel) from samples collected on day 21 and creatinine clearance was calculated according to the standard formula ((urinary creatinine (mmol)*1000)/plasma creatinine (µmol)*(urine volume/1440)).

Immunohistochemistry

After paraformaldehyde fixation, renal tissue was processed for paraffin embedding according to standard methods. For immunohistochemistry, 2µm sections were cut. Total macrophages/monocytes (ED-1) and M2-like macrophages were identified by staining for CD68 (1:100 diluted, clone ED1, AbD Serotec, Düsseldorf, Germany) and CD206 (1:1000 diluted; Abcam, Cambridge, UK) respectively according to standard methods [21]. The pre-fibrotic marker for myofibroblast transformation alpha-smooth muscle actin (αSMA) was detected using a murine monoclonal antibody (αSMA; clone 1A4; Sigma) as previously described [22].

Morphometric analysis of immunohistochemical staining

Interstitial ED-1 and CD206 positive cells were determined by manually analysing 30 randomly selected cortical fields per kidney (40x magnification), excluding fields with glomeruli in a view. For each cortical field, the number of positive cells was counted. Sections stained for αSMA were scanned using an Aperio ScanScope CS and analyzed with Aperio ImageScope v10.2.2.2319 (Aperio, Vista, CA, USA). The 'Positive pixel Count V9' algorithm was used to analyse αSMA-positive pixels after excluding vessels. The total positive surface area of all fields, was divided by the total area of all fields measured, providing a number of αSMA positive pixels corrected for the area analyzed. Researchers were blinded for group allocation of the rats while analysing the kidney slides.

mRNA expression analysis

Total aortic and kidney RNA was isolated with TRIzol Reagent (Invitrogen) following manufacturer's instructions. Total RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA synthesis was performed as described before [23]. Real time quantitative PCR was performed using Lightcycler 480 (Roche, Applied Science) and Applied Biosystems reagents according to the manufacturer's instructions. Expression levels were normalized to those of 18S ribosomal RNA, which was analysed in separate runs. Primers and probes for the AT1-R and AT2-R were obtained from Applied Biosystems (TaqMan Gene Expression Assays, AT1-R: Rn00578456_m1 and AT2-R: Rn00560677_m1). The sequences for 18S (M11188) (sense primer, antisense primer, and probe, respectively; all from 5' to 3') were: CGGCTACCACATCCAAGGA, CCAATTACAGGGCCTCGAAA, CGCGCAAATTACCCACTCCCGA.

Protein expression analysis

Kidney lysates (20 μg per lane) were run on 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidine difluoride membranes.
Membranes were rinsed with tris buffered saline (TBS) containing 0.05% Tween 20 (TBST) then blocked with TBST containing 5% bovine serum albumin (BSA) for 1-hour at room temperature (RT). Blots were then incubated with primary antibodies (AT1-R: ab124734 (Abcam), AT2-R: Mab 3659 (R&D systems, Inc, Minneapolis)) diluted in TBST containing 1% BSA overnight at 4 °C. The blots were then rinsed with TBST (3 times) for 5 min at RT and incubated with the appropriate secondary antibody (Polyclonal Goat anti Rabbit HRP; P0448 (Dako Netherlands bv) diluted in TBST containing 1% non-fat dry milk and 2% normal rat serum for 1-hour at RT. The blots were rinsed another 3 times for 5 min with TBST before visualization was done by enhanced chemiluminescence (ECL), according to standard procedures with a molecular imager geldoc XR system from Bio-Rad (Bio-Rad Laboratories, Inc). After that, blots were stripped by incubating in 25 mM Glycine, 2% SDS, (pH 6.7) for 30 min at RT. Stripped blots were then rinsed extensively with TBST and reprobed as described above with a β-Actin antibody (SC-47778 (Santa-Cruz Biotechnology, Inc.). Using the expression of β-Actin as reference, protein bands were quantified with Image-lab 4.0.1 from Bio-Rad. The ratio of AT1-R or AT2-R to β-Actin was calculated.

Aortic ring contraction experiment

Drugs and chemicals

Krebs buffer was freshly made before the start of each experiment and contained in mmol/L: 120 natrium chloride (NaCl), 5.9 potassium chloride (KCl), 25.2 $NaHCO_3^-$, 1.2 NaH_2PO_4, 10.4 glucose, 1.21 $MgCl_2 \bullet 6H_2O$, and 2.52 $CaCl_2$. All Krebs ingredients were purchased from E. Merck (Darmstadt, Germany). The stock solutions for phenylephrine (Sigma, St. Louis, MO, USA), ang II (Bachem AG, Bubendorf, Zwitzerland), PD-123319 (Park-Davis), Losartan (Merck Research laboratories, Rahway, USA), and N^G-nitro-L-arginine methyl ester (L-NMMA; Calbiochem Brand of EMD Biosciences, Inc., La Jolla) were prepared in saline (0.9%NaCl in distilled water).

Ex-vivo aortic reactivity to ang II

The *ex-vivo* sensitivity to ang II in aortic tissue was studied in isolated aortic rings of the c-NP, c-fHP, and c-fPE-rats, using standard isotonic contraction experiments as previously described [24]. Aortic rings (2mm) from the rats were kept in Krebs solution (at 37°C) and aerated with 95% CO_2 and 5% O_2. The aortic rings were equilibrated for 30 minutes before they were primed and checked for viability by evoking a contraction with KCl (60mM) for 10 minutes twice. To study the ang II sensitivity per se, a cumulative ang II concentration-response curve (10^{-10}M-10^{-6}M) was obtained. 10^{-5}M phenylephrine was added after completing the ang II concentration-response curve to assess total aortic ring contraction. The ang II-mediated contraction was then expressed as a percentage of the maximum contraction after 10^{-5}M phenylephrine. Response to ang II via the ang II type 1 (AT1-R) and/or type 2 receptor (AT2-R) were studied as described previously [24]. In short functional response of the AT1-R to ang II was studied after incubation with 10^{-6}M PD-123319 (AT2-R antagonist) and selective nitric oxide (NO) synthase inhibitor L-NMMA (10^{-4}M) to prevent any confounding effects by the basal release of NO [25]. Then, a cumulative ang II concentration-response curve (10^{-10}M-10^{-6}M) was obtained according to standard methods [26]. 10^{-5}M phenylephrine was added after completing the ang II concentration-response curve to assess total aortic ring contraction. The ang II-mediated contraction was then expressed as a percentage of the maximum contraction after 10^{-5}M phenylephrine. The functional response of the AT2-R to ang II was studied after incubation with 10^{-5}M losartan (AT1-R antagonist). After pre-contraction with 10^{-6}M phenylephrine, the cumulative ang II concentration-response curve (10^{-10}M-10^{-6}M) was obtained. The ang II-mediated relaxation was then expressed as a percentage of the maximum pre-contraction with phenylephrine.

Data analysis

Statistical analysis was performed using SPSS for Windows (Version 20.0) and area under the curve (AUC) was calculated using GraphPad Prism 5, on a standard computer. Parametric data are presented as mean ± standard deviation (SD) in text, table and figures, unless otherwise stated and analyzed using Student t-test. Nonparametric data are presented as median (25^{th}-75^{th} percentile) and analyzed using Mann-Whitney U. For multiple testing, One-way ANOVA followed by LSD post-hoc analysis was used. The increase in systolic blood pressure and proteinuria was calculated as the percentage increase three weeks after minipump implantation as compared to baseline systolic

blood pressure and proteinuria. To test for significant correlations, the Spearman's rho correlation test was used. In all cases, differences were considered significant if $p<0.05$.

RESULTS

Human study

Baseline characteristics of the women

There were no statistically significant differences in most of the characteristics between the two groups (Table 1). However, fPE-women had a significantly higher BMI and office blood pressure at intake, all within the healthy range. Importantly, 24-hour ABPM and baseline blood pressure at the study day were not different between the groups. Analysis of blood parameters revealed no differences (Table 2).

Table 1. Baseline characteristics

		fHP-women (n = 18)	fPE-women (n = 18)	p-value
Age (years)		36 ± 5	36 ± 5	0.951
Gravidity		2.5 ± 1.3	2.6 ± 1.1	0.951
Parity		2.0 ± 0.7	2.2 ± 1.0	0.589
Elapsed time since index pregnancy (years)		4.2 ± 2.6	5.3 ± 3.0	0.243
BMI (kg/m^2)		23.2 ± 2.7	25.9 ± 3.5	**0.015**
Waist/Hip ratio		0.83 ± 0.04	0.84 ± 0.06	0.443
Urinary sodium (mmol/24h)		221 ± 64	258 ± 86	0.145
Urinary albumin/creatinine		0.6 ± 0.3	0.5 ± 0.4	0.212
MAP (mmHg)	Screening visit[1]	90 ± 7	97 ± 8	**0.01**
	24-hour[2]	90 ± 7	90 ± 8	0.997
	Baseline[3]	85 ± 8	86 ± 9	0.714

fHP-women, formerly healthy pregnant women; fPE-women, formerly early-onset preeclamptic women; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. [1]Average of three blood pressure measurements assessed by the Dinamap during the screening visit in the hospital; [2]blood pressure measured using ambulatory blood pressure measurement for 24-hours at the end of the dietary week; [3]baseline blood pressure measured at study day, measured for 2 hours with a 15-min interval. Data are presented as mean ± SD.

Table 2. Blood parameters

	fHP-women (n = 18)	fPE-women (n = 18)	p-value
Hematocrit	0.38 ± 0.03	0.38 ± 0.03	0.634
Glucose (mmol/L)	5.0 ± 0.5	5.0 ± 0.3	0.605
HbA1c (mmol/mol)	35 (32.75-37.25)	34 (30.75-35.25)	0.203
Insulin (uU/mL)	7.1 (4.7-9.3)	7.65 (4.6-10.8)	0.525
HOMA[1]	1.55 (0.96-2.21)	1.69 (1.08-2.37)	0.369
Creatinin clearance (mL/min)[2]	113 ± 24	123 ± 25	0.220
eGFR (mL/min/1.73m^2)[3]	109 ± 10	103 ± 15	0.117
PRA (nmol Ang I/L/h)	0.20 (0.10-0.50)	0.20 (0.09-0.30)	0.584
Aldosteron (pmol/L)	71 (29-93)	59 (35-96)	0.839

*fHP-women, formerly healthy pregnant women; fPE-women, formerly early-onset preeclamptic women; HOMA, homeostatic model assessment index; eGFR, estimated glomerular filtration rate; PRA, plasma renin activity. [1]Calculated using the formula: (glucose*insulin)/22.5; [2]calculated using the formula: ((urinary creatinine excretion/24h * 1000)/plasma creatinine); [3]calculated using the CKD-epi formula [18]. Data are presented as mean ± SD as median (25th-75th percentile).*

Increased blood pressure response to ang II infusion in fPE-women

In response to 1.0 ng/kg/min ang II infusion, a trend towards an increased DBP response upon ang II in fPE-women as compared to fHP-women was present. DBP after 0.3 and 3.0 ng/kg/min ang II was not different between both groups (Figure 1B). Similar results were found for MAP responses upon ang II infusion (Figure 1C). No significant differences in SBP response were observed between the two groups during the subsequent ang II infusion doses (Figure 1A). Heart rate was not significantly different after ang II infusion between the two groups (data not shown). Studying the AT1-AA showed that 44.4% of the fPE-women and 16.7% of the fHP-women were positive for AT1-AA (8 vs 3 respectively; p=0.070). Serum from fPE-women increased the overall cardiomyocyte-beating rate, although not significant compared to fHP-women (median change in bpm: fPE-women: 6.4 (0.5-16.7) vs fHP-women: 0.68 (0-5.4); p=0.139). No significant correlation was found between the presence of AT1-AA and the degree ang II sensitivity or time after index pregnancy (data not shown).

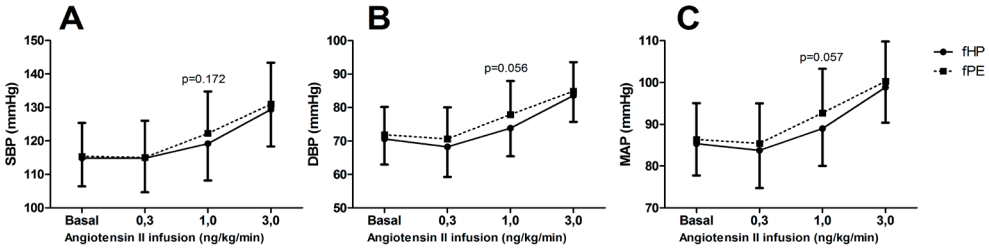

Figure 1. Blood pressure response upon angiotensin II infusion in women.

The mean (±SD) of the systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (C) at baseline and after 0.3, 1.0, and 3.0 angiotensin II (ng/kg/min) respectively as compared to baseline in fHP-women (circle) and in fPE-women (square, dotted line). Data were analyzed using the general estimating equations and corrected for baseline blood pressure and BMI. fPE: formerly preeclamptic women; fHP: formerly healthy pregnant women. P-values are presenting the difference between the two groups at 1.0ng/kg/min angiotensin II.

Animal study

Rat characteristics

At the end of pregnancy, HP-rats weighed significantly more compared to the PE-rats (Table 3A). The total number and weight of pups was not significantly different, however, the length of the pups was significantly decreased in PE-rats as compared to HP-rats. Moreover, one pup from one PE-rat and eight pups from another PE-rat were born dead (Table 3B). Baseline SBP, i.e. six weeks after delivery, was slightly but significantly lower in the fPE-rats as compared to the NP-rats. No difference in baseline proteinuria was found between the three groups.

Increased blood pressure response upon ang II infusion in fPE-rats

In the control rats, i.e. rats treated with sham pumps, no significant changes in SBP were observed in the three weeks of treatment (not shown). However, SBP increased in all groups of rats following three weeks of continuous ang II infusion (Figure 2A), with the highest increase in A-fPE-rats. The percentage increase in SBP in A-fPE-rats was higher (borderline significant), as compared with A-NP-rats (Figure 2A). SBP increase after one and two weeks of ang II infusion revealed no differences between the three groups of rats (not shown). SBP at time of termination (under anesthesia) was significantly higher in A-fPE-rats as compared to A-NP-rats (Figure 2B), with no significant differences between the three groups of sham treated rats (not shown).

Table 3. Rat characteristics during pregnancy (A) and postpartum (B)

A - PREGNANCY CHARACTERISTICS			
	HP-rats (n = 19)	**PE-rats (n = 22)**	**p-value**
Rat maternal weight[1] (g)	383 ± 24	361 ± 26	**0.013**
Number of pups[2]	12.4 ± 3.4	10.7 ± 3.9	0.150
Length of pups[2] (mm)	50.6 ± 1.4	49.2 ± 2.7	**0.034**
Weight of pups[2] (g)	6.1 ± 0.3	6.0 ± 0.8	0.556
B - POSTPARTUM CHARACTERISTICS			
	NP-rats (n = 25)	**fHP-rats (n = 19)**	**fPE-rats (n = 22)**
Rat maternal weight[3](g)	269.7 ± 17.9	274.9 ± 17.8	276.4 ± 12.8
Baseline SBP[4] (mmHg)	129.9 ± 9.5	127.5 ± 9.8	122.9 ± 9.1*
Baseline proteinuria[4] (mg/24h)	4.3 ± 1.2	4.2 ± 2.3	3.3 ± 1.8

*HP, healthy pregnant; PE, experimental preeclampsia; fHP, formerly healthy pregnant; fPE, formerly experimental preeclampsia; NP, never pregnant; SBP, systolic blood pressure. [1]Weight at the day before delivery; [2]only pups that were moving were measured; [3]weight at the time of osmotic minipump implantation (six weeks postpartum); [4]baseline values measured six weeks postpartum (fHP- and fPE-rats) or at the same time-interval for the NP-rats. Data are presented as mean (± SD). *: p<0.05 vs NP-rats.*

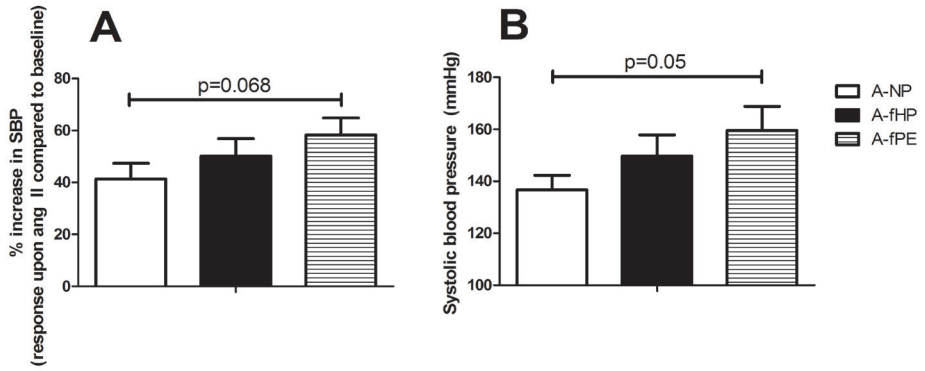

Figure 2. Blood pressure response upon chronic angiotensin II infusion in rats.

(A) The mean ± SEM of the percentage increase in systolic blood pressure (SBP) after three weeks of 200ng/kg/min angiotensin II (ang II) compared to baseline SBP in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Blood pressure was measured by the use of a tailcuf device. Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. (B) The mean ± SEM of the SBP at termination after three weeks of 200ng/kg/min ang II in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis.

Changes in sensitivity of the thoracic aorta to ang II: aortic ring contraction experiment

Ex-vivo ang II sensitivity was studied in aortic rings in the sham treated rats. Although the area under the curve (AUC) showed no significant differences between the three groups (Figure 3 A1 and A2), $logEC_{50}$, was significantly lower in the c-fHP-rats as compared with c-fPE-rats (Table 4). No significant difference in AT1-R mediated vasoconstriction was observed between the three groups of rats (Figure 3 B1 and B2). It can be seen that AT2-R mediated relaxation, as measured by AUC (area of the positive peaks) was significantly impaired in the c-fPE-rats as compared to the c-NP-rats (Figure 3 C2).

Significant increase in proteinuria following ang II infusion in fPE-rats

No significant increase in proteinuria over the three weeks of treatment was seen in rats that received a sham osmotic minipump (not shown). After three weeks of continuous ang II infusion, proteinuria increased in all three groups, with a trend towards increased proteinuria in fPE vs fHP-rats (data not shown). At this time point, the percentage increase in proteinuria as compared to baseline was significantly higher in A-fPE as compared to A-fNP and A-fHP (Figure 4A). Creatinine clearance (mL/min) was not significantly different between the rats implanted with a sham osmotic minipump (cNP 2.5 ± 0.5 vs c-fHP 2.8 ± 0.7 vs c-fPE 2.5 ± 0.7; p = 0.408). Also no difference was seen between the rats receiving ang II (A-NP 2.2 ± 0.6 vs A-fHP 2.3 ± 0.5 vs A-fPE 2.3 ± 0.4; p = 0.859).

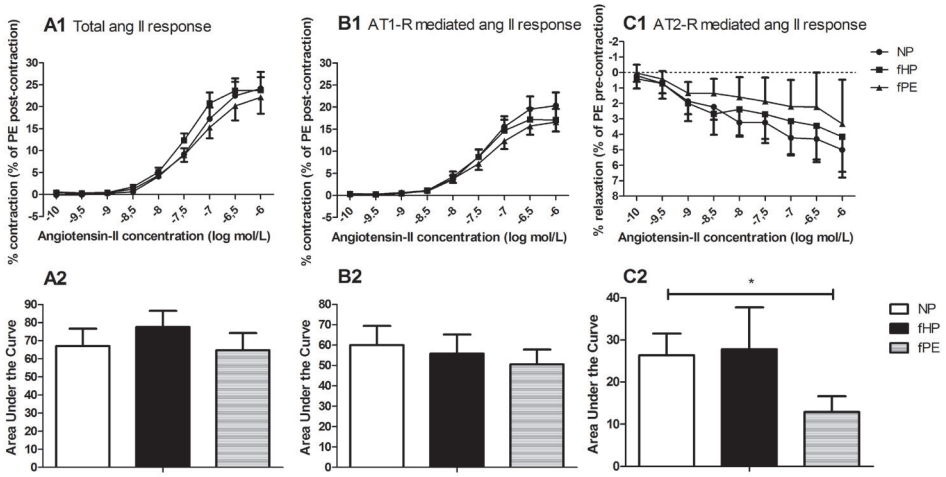

Figure 3. Ang II dose-response curves in the thoracic aorta.

*The mean ± SEM of the cumulative angiotensin II (ang II) dose-response curves in the thoracic aorta from never pregnant rats (NP; circle; n=12), formerly healthy pregnant rats (fHP; square; n=10), and formerly experimental preeclampsia rats (fPE; pyramid upward; n=11). (A) ang II was added with no inhibitor present; (B) ang II was added after incubation with the AT2-R antagonist PD123319 and L-NMMA; (C) ang II was added after incubation with the AT1-R antagonist losartan. (A2, B2, C2): mean ± SEM AUC of the cumulative ang II dose-response curves in never pregnant rats (NP; white bar), formerly healthy pregnant rats (fHP; black bar), and formerly experimental preeclampsia rats (fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. *:p<0.05*

Table 4. logEC50 and Emax dose response curves

	c-NP-rats (12)	c-fHP-rats (10)	c-fPE-rats (11)
log EC_{50}			
Ang-II	-7.29 (± 0.18)	-7.45 (± 0.23)	-7.25 (± 0.24)*
Ang-II contraction	-7.29 (± 0.30)	-7.42 (± 0.24)	-7.29 (± 0.28)
Ang-II relaxation[1]	-8.11 (± 1.04)	-8.66 (± 0.62)	-8.13 (±1.14)
E_{max}			
Ang-II	26.06 (± 13.59)	25.81 (±9.62)	23.93 (± 13.09)
Ang-II contraction	22.14 (± 10.54)	18.64 (± 8.27)	17.84 (± 7.08)
Ang-II relaxation[1]	4.60 (± 5.00)	6.31 (± 9.77)	4.80 (±10.11)

*c-NP, control never pregnant rats; c-fHP, control formerly healthy pregnant rats; c-fPE, control formerly experimental preeclampsia rats. [1]for fPE group only 7 rats were included in analyses. Data are presented as mean ± SD. *: p <0.05 vs c-fHP.*

Increased inflammation of the kidney in fPE-rats in response to ang II

Ang II infusion did not affect the number of interstitial monocytes/macrophages in NP and fHP-rats. However, three weeks after ang II infusion of fPE-rats the amount of interstitial monocytes/macrophages was significantly higher as compared to sham treated fPE-rats (Figure 4B). No differences were seen after sham or ang II infusion between NP-, fHP-, and fPE-rats in the number of CD206 positive macrophages (data not shown). In all groups, treated for three weeks with ang II, we found a significant increase in the number of positive pixels per area for αSMA as compared to the sham treated rats (data not shown), with no differences between these groups.

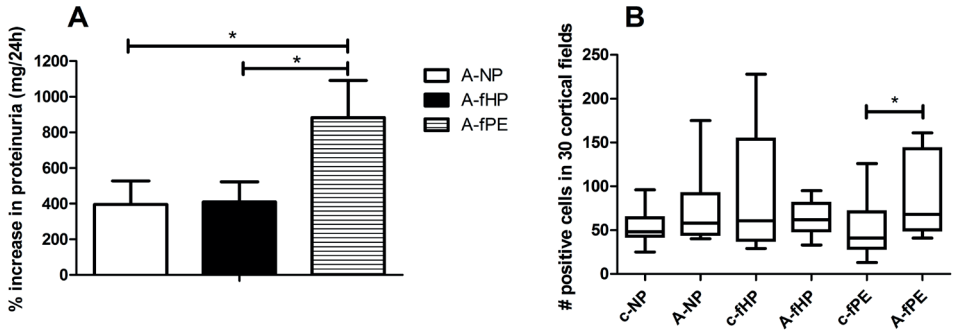

Figure 4. Proteinuria and macrophages influx upon chronic angiotensin II infusion.

*(A) The mean ± SEM of the percentage increase in proteinuria (mg/24h) after three weeks of 200ng/kg/min angiotensin II (ang II) compared to baseline proteinuria in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. *: p<0.05*

*(B) The median with 25th-75th percentile of the number of CD68-positive macrophages (ED1-macrophages) in the interstitial part of the kidney and in sham operated never pregnant rats (c-NP), formerly healthy pregnant rats (c-fHP) and, formerly preeclamptic rats (cfPE). and in never pregnant rats (A-NP), formerly healthy pregnant rats (A-fHP), and formerly preeclamptic rats (A-fPE) infused with 200ng/kg/min ang II for three weeks. Data were analyzed using Mann-Whitney U test. *: p<0.05*

AT1- and AT2-receptor mRNA expression and protein expression in the aorta and kidney

The increased blood pressure and proteinuria response upon infusion of ang II could be due to a different regulation of the AT1-R and AT2-R upon chronic ang II infusion between the three groups. Therefore, the mRNA expression of the AT1-R and AT2-R in the thoracic aorta and the kidneys of all groups were measured. We observed a trend towards an increased mRNA expression of the AT1-R (Figure 5A) in the thoracic aorta of the A-fPE-rats as compared to c-fPE-rats. This effect of chronic ang II infusion was not observed in ang II treated NP-rats and fHP-rats. No differences were seen in AT2-R mRNA expression in the thoracic aorta between the different groups (Figure 5B). No difference in mRNA expression for the AT1-R and the AT2-R was found between the three groups of rats with sham pumps.

The mRNA expression of the AT1-R and the AT2-R in the kidney of sham treated rats was comparable between the three groups. Moreover, no effect of chronic ang II infusion upon expression of either AT1-R mRNA or AT2-R mRNA was observed. However, after chronic ang II infusion, AT1-R mRNA expression was significantly lower in the fPE-rats as compared to the NP-rats, with no differences in AT2-R mRNA expression (Figure 5C and Figure 5D).

Protein expression of the AT1-R in the kidney of sham treated rats was comparable between the three groups (Figure 5E). After chronic ang II infusion, AT1-R protein expression showed a trend towards a lower expression in the fPE-rats as compared to the NP-rats (Figure 5E). Very low levels of AT2-R protein expression were found in the kidney extracts. However, AT2-R protein expression was significantly increased in sham treated fPE-rats as compared to sham treated fHP-rats (Figure 5F). Also, after chronic ang II infusion, protein AT2-R expression was significantly increased in fPE-rats as compared to fHP-rats (Figure 5F). Unfortunately, due to a lack of aorta material, we were not able to study AT1-R and AT2-R protein expression in the aorta.

DISCUSSION

This is the first translational study that analyses ang II sensitivity in formerly early-onset preeclamptic women without any comorbidity and in healthy rats after experimental preeclampsia. We demonstrated increased blood pressure response to ang II infusion, albeit subtle (borderline significant) in formerly early-onset preeclamptic women as compared with formerly healthy pregnant women. Moreover, after experimental preeclampsia we observed an increased blood pressure, proteinuria and macrophage influx into the kidney after chronic infusion of ang II as compared with formerly healthy pregnant rats. Since we studied women without any comorbidity and rats that were healthy and identical pre-pregnancy, our data suggest that (experimental) preeclampsia itself may have induced the persistent increased ang II sensitivity in the present studies. This increased ang II sensitivity postpartum might be one of the pathophysiological mechanisms leading to the increased risk for cardiovascular and renal diseases in fPE-women.

Blood pressure appeared to increase more in response to ang II in fPE-women compared to fHP-women, however, this was just not significant. Our data appear to be in line with a study of Hladunewich *et al*., however, they found more pronounced differences. This may be due to the fact that they studied women 6-18 months postpartum rather than 1-10 years postpartum [11]. Furthermore, their study group showed increased baseline blood pressure, BMI and age compared with controls. Similarly, differences between our study and the study of Spaanderman *et al*. [27] and Saxena *et al*. [10] may be explained by differences in study design, such as a mixture of phenotypes and increased blood pressure in the study group, the ang II concentration and low sodium intake used, and no standardization of menstrual cycle in both studies. Although in the present study, the fPE-women showed a higher BMI, the waist/hip ratio, which is a superior risk factor for cardiovascular disease than BMI [28], was not different between the groups. The white-coat effect observed in our study may be related to the increased risk for cardiovascular disease [29].

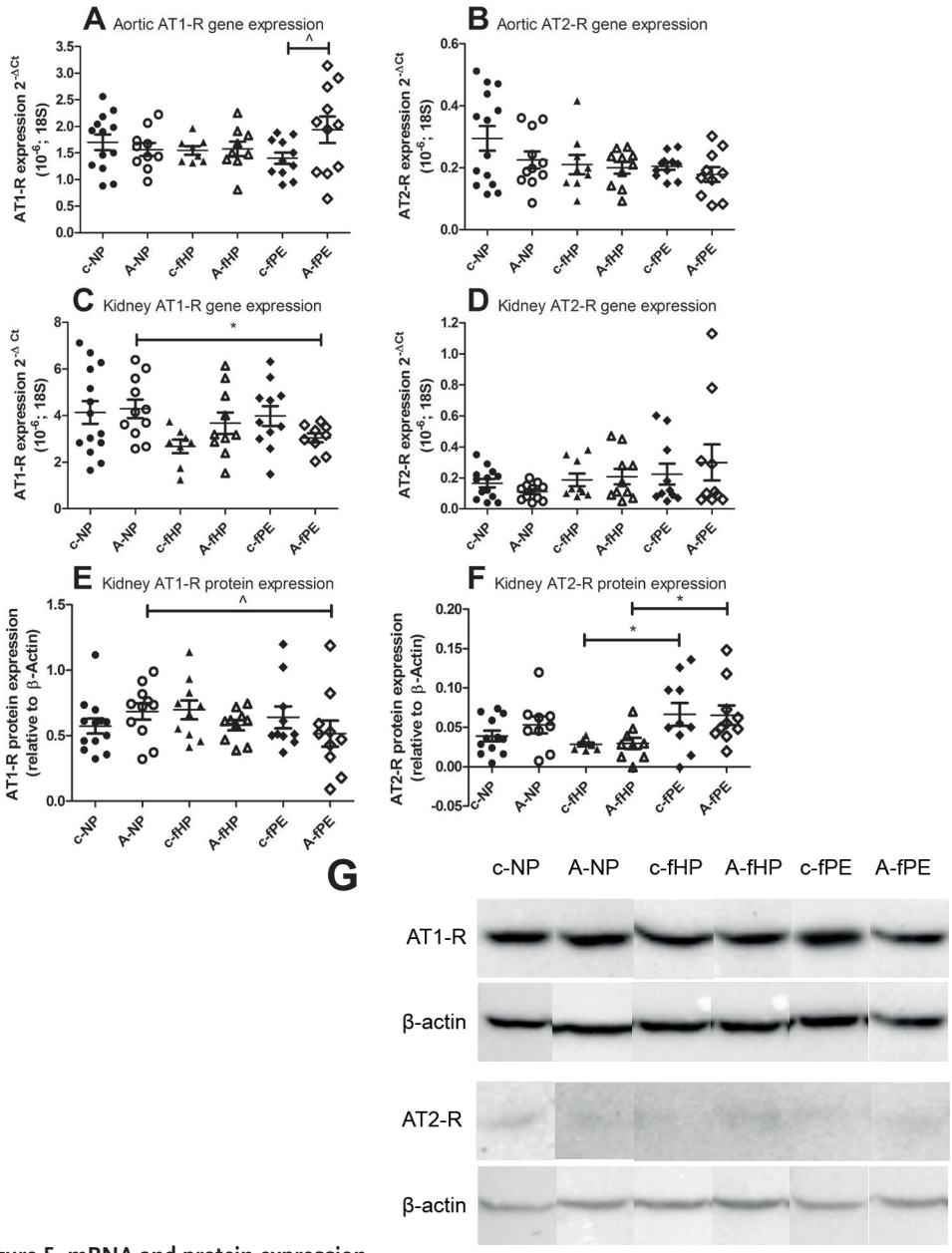

Figure 5. mRNA and protein expression.

*The mRNA expression of the AT1-R (A, C) and the AT2-R (B, D) in the thoracic aorta (A, B) and the kidney (C, D) and the protein expression of the AT1-R (E) and the AT2-R (F) in the kidney in sham operated never pregnant rats (c-NP), formerly healthy pregnant rats (c-fHP), and formerly preeclamptic rats (c-fPE), and in never pregnant rats (A-NP), formerly healthy pregnant rats (A-fHP), and formerly preeclamptic rats (A-fPE) infused with 200ng/kg/min angiotensin II for three weeks. Representative western blot analysis of AT1-R and AT2-R and the associated β-actin protein expression in the kidney in the 6 groups of rats (G). Data were analyzed using Mann-Whitney U test. *: p<0.05, ^: p<0.1*

To investigate cause and effect relationships between preeclampsia and postpartum ang II sensitivity in a clean model, we studied blood pressure and kidney response to chronic ang II infusion after experimental preeclampsia, induced by infusion of low dose LPS [12]. Previous studies have shown that LPS-infused pregnant rats develop a preeclampsia like syndrome, as characterized by hypertension and proteinuria [12, 30, 31]. Since the sensitivity to ang II is increased in this experimental preeclampsia model during pregnancy [24], this appears to be an appropriate model to study the relationship between the increased ang II sensitivity during and after preeclampsia. In accordance with our human data, in fPE-rats we found no baseline hypertension or proteinuria. In fact, fPE-rats even showed a significantly lower SBP before implantation of the minipumps, compared with the NP-rats. This might be related to the decreased vascular response to ang II found in the aorta of these fPE-rats (see below). Still, three weeks after chronic ang II infusion we observed an increased blood pressure and proteinuria response upon ang II in fPE-rats as compared to NP-rats and fHP-rats. Our data are in line with a study in preeclamptic mice (due to overexpression of soluble fms-like tyrosine kinase-1 (sFlt-1)), in which no long-term differences in blood pressure and vascular function were observed [32], while different expression of plasma proteins involved in cardiovascular function was seen, indicating postpartum effects induced by experimental preeclampsia [33]. In our animal model, preeclampsia-like symptoms are induced by LPS via activation of Toll-like receptor [4]. Many other animal models for preeclampsia exist, for instance overexpressing sFlt-1 [34], ATP-infusion [35], and renin-angiotensin aldosterone system (RAAS) overactivity [36] or genetic adjustments [37], [38]. In these models, the preeclampsia-like symptoms may be induced via other pathways. Therefore, our animal data should be confirmed in other models of preeclampsia.

To test *ex-vivo* ang II sensitivity in our rat model, we used the easily accessible aorta, and performed aortic ring contraction experiments in response to ang II. The aorta is a conductance vessel rather than a resistance vessel. The aorta is therefore not typically involved in blood pressure regulation. However, the use of aortic rings is a well-established read-out for vascular pharmacological responses, including studying angiotensin sensitivity. Although the total response to ang II (i.e. AUC) did not differ between the groups, $\log EC_{50}$ was significantly lower in the fHP-rats. This suggests, in contrast to our hypothesis, a decreased sensitivity to ang II in the aorta of fPE-rats as compared to fHP-rats. The question arises whether this decreased sensitivity in fPE-rats is related to the slightly decreased blood pressure measured in these rats at baseline. It may be speculated that the decreased ang II sensitivity in the aorta may decrease vascular smooth muscle tone and thus aortic stiffness and therefore central blood pressure [39] and possibly peripheral blood pressure. Further studies in other vascular beds, such as small resistance or renal arteries are needed to confirm that changes in the ang II sensitivity in fPE-rats are linked to blood pressure.

The ang II response via the AT2-R showed a decreased relaxation upon ang II in fPE-rats. This was, however, not accompanied by changes in the mRNA expression of the AT2-R in the aorta. The decreased *ex-vivo* responsiveness of the AT2-R to ang II in the fPE-rats might contribute to the increased blood pressure response upon ang II. This hypothesis, however, needs to be confirmed in resistance vessels. In contrast to differences in AT2-R responses in fPE-rats, we did not find differences in the AT1-R responses in fPE-rats. This is in contrast to the situation

during experimental preeclampsia, in which the response of the aorta to the AT1-R was increased [24]. Thus, although we have observed changes in ang II sensitivity during and after preeclampsia in the rat, during preeclampsia the AT1-R may be involved, while after preeclampsia the AT2-R may be involved. Further postpartum follow-up studies, also in other models for preeclampsia, are needed to establish the time course of changes and mechanisms in the AT1-R and AT2-R sensitivity.

We also observed increased proteinuria in response to ang II infusion in fPE-rats without differences in serum and urinary creatinine. This rise in urinary protein level was associated with an increased influx of interstitial macrophages. Since we found no increase in the number of CD206 positive macrophages, i.e. M2 macrophages, the increased macrophages are most likely of the M1 or inflammatory phenotype. It can be suggested that the increase in interstitial macrophages was secondary to proteinuria, since excessive tubular reabsorption of proteins results in tubulo-interstitial infiltration of monocytes [40]. As macrophages themselves may also inflict kidney injury [41], this macrophage infiltration may induce a vicious circle of kidney damage leading to proteinuria. On the other hand, as infiltration of macrophages in glomeruli and renal interstitium sometimes precedes the onset of glomerular injury and interstitial fibrosis leading to proteinuria [42], the infiltrated macrophages may also be the cause of proteinuria. Profibrotic changes, as measured by αSMA expression, were similarly increased in all groups after ang II infusion. Whether infusion duration or higher dosages of ang II would have resulted in more pronounced changes in renal damage in fPE-rats (i.e. focal glomerular sclerosis) as compared to the other groups, remains to be investigated.

The mechanism of increased ang II sensitivity during and after preeclampsia is unknown. The study of Hladunewich *et al*. suggested that the balance between the AT1-R and AT2-R could be involved [11]. Indeed, our data suggest differential expression and regulation of the AT1-R and AT2-R with ang II infusion in fPE-rats as compared with the other groups of rats. However, the present results for AT1-R and AT2-R mRNA and protein expression are not conclusive, since differential responses of the aorta and kidney were observed. Other RAAS mechanisms like, heterodimerization of the AT1-R with the bradykinin B(2)-receptor [43] or alterations in the vasodilatory ang 1-7 acting on the MAS-receptor [44] could be involved and should be studied in more detail. Furthermore, increased levels of AT1-AA postpartum could be involved in increased ang II sensitivity [17, 45]. Indeed in our study, although just not significant, more fPE-women tested positive for having AT1-AA as compared to fHP-women, but not correlating with ang II sensitivity. It seems unlikely that this contributes in our experimental rat model since we did not find AT1-AA in pregnant LPS-infused rats (unpublished results) .

Conclusion Our translational study suggests that preeclampsia per se leads to subtle long-term increased blood pressure and proteinuria in response to acute and chronic ang II infusion in otherwise healthy humans and rats. However, we cannot rule out that underlying pre-pregnancy (risk) factors may also play a role. Preeclampsia might merely identify women with an unfavorable cardiovascular system or aggregation of cardiovascular risk factors might occur before and during the preeclamptic pregnancy.

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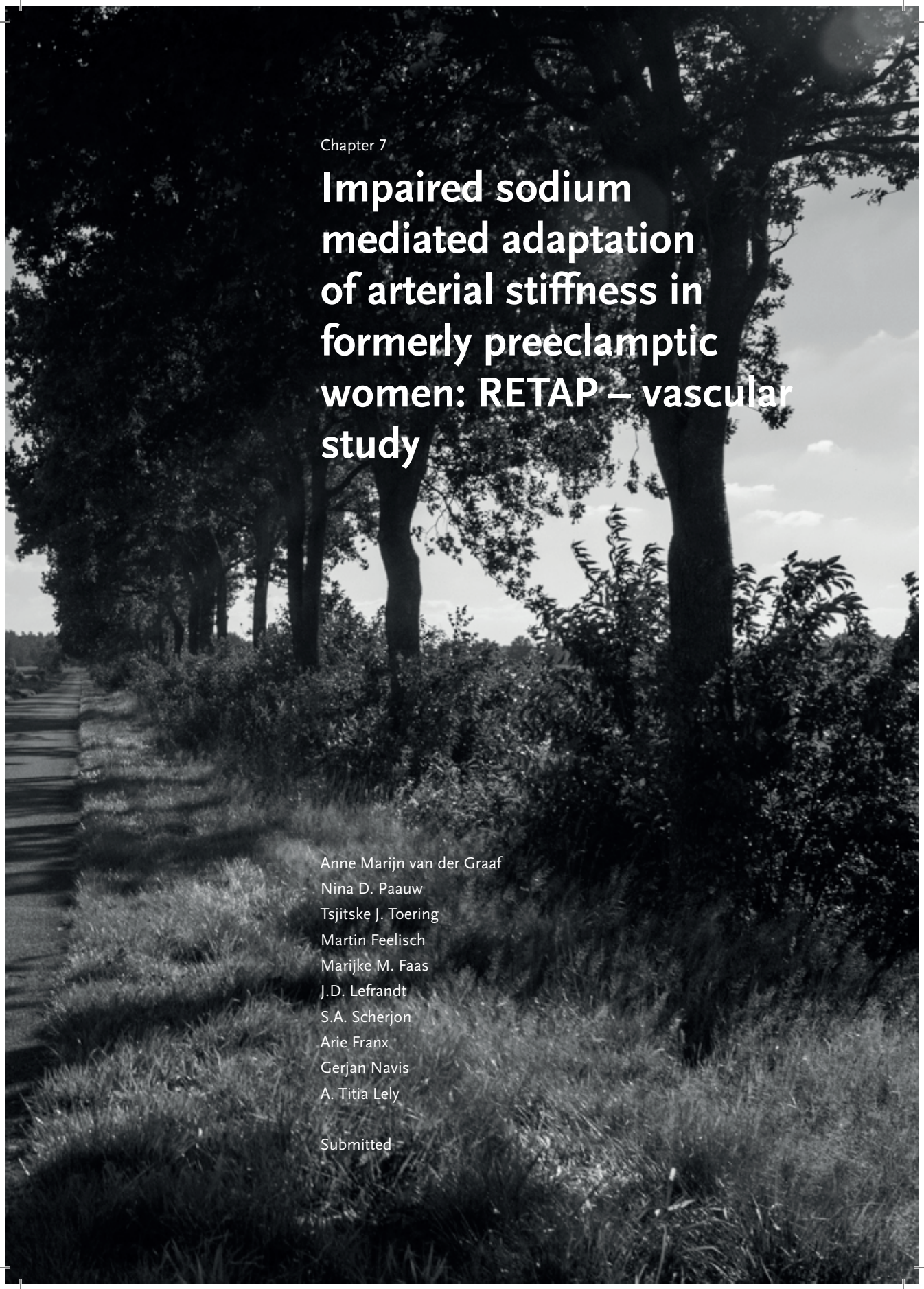

Chapter 7

Impaired sodium mediated adaptation of arterial stiffness in formerly preeclamptic women: RETAP – vascular study

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ABSTRACT

Objectives

Women with a history of preeclampsia have an increased risk for cardiovascular diseases later in life. Persistent vascular alterations in the postpartum period might be a potential mechanism behind this increased risk. This study aimed to assess arterial stiffness under low sodium (LS) and high sodium (HS) conditions in a healthy well-characterized group of formerly early-onset preeclamptic (fPE) women compared to formerly healthy pregnant (fHP) women.

Methods

18 fHP and 18 fPE women were studied in balance on one week of LS (50 mmol Na^+/day) and one week of HS (200 mmol Na^+/day) intake. Arterial stiffness was measured by augmentation index (AIx), AIx corrected for heart rate (AIx@75) and pulse wave velocity (PWV). Circulating parameters of renin-angiotensin aldosterone system (RAAS) activity, extracellular volume (ECV), nitrate, nitrosated species (RxNO) and cyclic GMP (cGMP) were measured to identify pathways underlying adaptation of arterial stiffness.

Results

As compared to HS diet, AIx and AIx@75 upon LS diet were significantly decreased in fHP women while there was no effect of LS consumption on AIx and AIx@75 in fPE women. PWV was similar in both groups and between diets. In both groups, comparable sodium dependent changes in RAAS, ECV and nitrate and were observed.

Conclusions

fPE women have an impaired ability to adapt their arterial stiffness (AIx/AIx@75) upon low sodium. The non-adaptation of arterial stiffness occurred independent of blood pressure, RAAS, ECV, and nitrates. The pathways involved in impaired adaptation, and its possible contribution to the increased long term risk for cardiovascular diseases in fPE women remains to be investigated.

INTRODUCTION

Preeclampsia complicates approximately 1-5% of the all pregnancies [19] and is characterized by new-onset hypertension and proteinuria during the second half of pregnancy [1]. Delivery of the placenta is the only therapeutic solution and results in rapid normalization of the maternal manifestations [44]. Despite normalization of hypertension and proteinuria after termination of pregnancy, a history of preeclampsia entails long-term vascular consequences. Over the years, observational cohort studies have shown that women with a history of preeclampsia experience an increased risk to develop premature cardiovascular and renal diseases in later life [3, 50, 52].

Unravelling the underlying mechanisms for the increased cardiovascular and renal risk after preeclampsia has been subject of recent studies. The increased risk could be the result of pre-existing cardiovascular risk factors as well as long-term effects caused by preeclampsia itself [36, 40, 49]. Since preeclampsia affects the maternal vascular bed, persistent vascular alternations in the postpartum period might be a potential mechanism for the increased cardiovascular risk in formerly preeclamptic women.

Recent studies in formerly preeclamptic women have reported subtle vascular alternations such as arterial stiffness as measured by pulse wave analysis (PWA) and pulse wave velocity (PWV)[10, 11, 24, 27, 31, 35, 37, 45, 53]. However, these studies show some inconsistencies; differences in study design, heterogeneity of the preeclamptic phenotype, and the presence or absence of comorbidities (i.e. hypertension, and increased BMI) may explain these contradictory findings. Moreover, none of these studies were performed under standardized sodium intake. Sodium restriction has been reported to be an important extrinsic factor in the reduction of blood pressure and arterial stiffness by volume reduction and reducing oxidative stress [15, 25, 42]. High sodium intake affects arterial stiffness by the induction of endothelial dysfunction, stimulation of vascular smooth muscle tone and hypertrophy of vascular wall [9, 17]. To our knowledge, adaptation or non-adaptation of arterial stiffness by short term changes in dietary sodium has not been studied in healthy subjects at risk for development of premature vascular diseases.

In this study, we aimed to explore arterial stiffness in the healthy formerly early-onset preeclamptic (fPE) women and formerly healthy pregnant (fHP) women in the absence of comorbidity. For this purpose, we measured arterial stiffness in the population of the Response to Angiotensin II in Formerly Preeclamptic women (RETAP) study of which we previously reported on renal function (RETAP-renal) [46]. All women were studied after one week of low sodium (LS) and after one week of high sodium (HS) intake to assess the influences of short-term changes in sodium diet on arterial stiffness. To identify pathways involved in the adaptation of arterial stiffness in response to salt we measured circulating components of the renin-angiotensin aldosterone system (RAAS), extracellular volume (ECV; ^{125}I-iothalamate distribution volume) and the endothelial function markers nitrate, nitrosated species (RxNO) and cyclic guanosine cyclic monophosphate (cGMP).

MATERIALS AND METHODS

Study population

Our study population consisted of 18 formerly early-onset preeclamptic women and 18 previously healthy pregnant controls who participated in the Response to Angiotensin II in Formerly Preeclamptic women (RETAP) study (The Netherlands National Trial Register www.trialregister.nl; trial registration number: 2635). Baseline characteristics of this study group and data on renal function and renal response to angiotensin II infusion within this group were published before (RETAP-renal) [46]. In short, this study showed no differences in GFR (measured by ^{125}I-iothalamate (IOT)) and no differences in renal hemodynamic response to angiotensin II infusion between groups, but the study did reveal a higher filtration fraction (FF) in the fPE group on both LS and HS diet.

The study population was selected from the electronic delivery database of the department of Obstetrics and Gynecology at the University Medical Center Groningen. Preeclampsia was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy [2] and early-onset preeclampsia was defined as developing preeclampsia before 34 weeks of gestation. Participants without comorbidity were selected by the exclusion of women with renal disease, diabetes or a history of gestational diabetes, obesity (BMI>30 kg/m^2 at screening) and women using any antihypertensive medication. Additional exclusion criteria were pregnancy, current lactation and post-menopausal status. None of the included women were using oral contraceptives. The formerly preeclamptic women were matched for age and year of index pregnancy (within one year) with a parous control whose pregnancy had been uncomplicated and normotensive. All subjects were non-smokers and normotensive, having a sitting systolic blood pressure <140mmHg and diastolic blood pressure <90mmHg measured by Dinamap (the average of three measurements was taken). All patients underwent physical examination and electrocardiography at intake of the study, which did not reveal any abnormalities. The study was approved by the local medical ethical committee (Medical Ethical Committee UMCG Groningen, the Netherlands; number 2010/294) and all subjects gave written informed consent.

Study protocol

The selected participants underwent a randomized cross-over protocol consisting of two one-week periods with at least four weeks in between, a 7-day period on LS diet (aim: 50 mmol Na^+/day) and a 7-day period on a HS diet (aim:200 mmol Na^+/day). For assessment of dietary compliance and the achievement of stable sodium balance, 24-hour urine was collected at day 3 and day 6 during each period. All women were studied at day 7 of each treatment after an overnight fasting period at day 7 ± 2 of the menstrual cycle.

Blood pressure and arterial stiffness measurements

Blood pressure was assessed at the end of each dietary period. After a two-hour rest in semi-supine position in a quiet room, blood pressure and heart rate were measured by the use of an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA)

at 15-min intervals for two hours (10am till 12am). Arterial stiffness was measured using the Sphymocor System. To obtain the augmentation index (AIx), recording of the radial pulse wave contour was performed by using applanation tonometry. In short, the artery of interest was pressed gently at the site of maximal pulsation with the tip of the tonometer containing a micromanometer that accurately records the pressure within the artery (Millar Instruments, Houston, TX). First a successive recording of the pressure waveform at the right brachial artery was assessed. These values were entered in the program (SphygmoCor; AtCor Medical, Sydney; version 8.2) and subsequently three successive recordings were performed on the right radial artery. The SphygmoCor software incorporates a quality control feature (operator index) which is displayed on the screen. An operator index above 80 was called a successive reading. Peripheral AIx is defined as the ratio of late systolic pressure (P2) to early systolic pressure (P1). AIx and AIx corrected for heart rate (AIx@75) were automatically calculated by the Sphygmocor. The average of the three successive readings was used in the analysis. The SphygmoCor system was subsequently used to assess carotid-femoral pulse wave velocity (PWV). The PWV was determined by sequential acquisition of pressure waveforms from the carotid and the femoral arteries. The timing of these waveforms was computed with that of the R-wave on the simultaneously recorded ECG. To reduce the influence of body contour, the proximal distance was measured from the sternal notch to the sampling site on the carotid artery and the distal distance was measured from the acromial angle to the sampling site on the femoral artery. The average of more than 8 successive measurements was used in the analysis to cover a complete respiratory cycle.

Extra-cellular volume measurements

ECV was estimated from the distribution volume of IOT. Assessment of ECV by the constant infusion method with IOT was validated and the method was demonstrated to be reproducible [51]. A priming solution containing 20 ml infusion solution (0.04 MBq) plus an extra amount of 0.6 MBq IOT was given at a constant infusion of 12 ml/h. Plasma concentrations of IOT were stabilized during 1.5-h equilibration and was followed by a 2-h period of clearance. ECV was calculated as follows: $[(I \times V + B \times V) - (U \times V)]/P$, where $I \times V$ is the infusion rate of the tracer, $B \times V$ the bolus infusion of the tracer and $U \times V$ the urinary excretion of the tracer [51]. This formula equals the amount infused IOT minus the amount excreted IOT. ECV was indexed for body surface area (BSA) by dividing the crude values by BSA multiplying it by 1.73 m^2. BSA was calculated according to the DuBois-DuBois formula [8]. Data of uncorrected ECV in this population were published earlier in the RETAP-renal manuscript [46].

Blood and urine sampling and analysis

Fasting blood samples were drawn for analysis of renin-angiotensin aldosterone system (RAAS) activity and endothelial function markers. Aldosterone was measured with a commercially available radioimmunoassay kit (Coat-A-count RIA, Siemens). PRA was measured with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess endogenous angiotensinogen (nanograms of angiotensin I produced per liter of plasma per hour; CisBio International, France). Plasma cGMP levels were assessed using a competitive enzyme immunoassay

(ELISA) according to the manufacturer's instructions (KGE003; R&D Systems, Minneapolis, MN). The concentration of the total pool of nitrosated species (RxNO) and nitrate were measured in the laboratory of M. Feelisch, University of Southampton. RxNO was assessed using group-specific reductive denitrosation by iodine-iodide in glacial acetic acid, with subsequent detection of liberated NO into the gas phase by its chemiluminescent reaction with ozon [12]. Plasma nitrates were quantified by ion chromatography with reduction of nitrate to nitrite and post-column Griess diazotization (ENO20 Analyser; Eicom, Kyoto, Japan) [34]. Urine samples were drawn from the 24-hour urine and the levels of sodium, potassium and urea were assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel).

Data analysis

Statistical analysis was performed using SPSS for Windows (Version 21.0). Parametric data are presented as means ± standard deviation (SD) or Estimated Marginal Means (EMM) ± standard error (SE) and non-parametric data as medians with interquartile ranges such as stated in text, table and figures. PWV values were log transformed. Generalized Estimated Equations (GEE) analysis was performed for AIx, AIx@75, PWV, and ECV to separately test the effects of history of preeclampsia (factor group) and sodium intake (factor diet). In addition, this analysis enabled us to separately study the changes in parameters in response to change in diet within the fHP and fPE group. The same GEE analysis was performed to analyze nitrate, RxNO, and GMP. To determine whether age is a determinant of arterial stiffness we used linear regression, which was performed for both LS and to HS diet. Differences were considered significant if $p<0.05$.

RESULTS

Baseline characteristics

Baseline characteristics of our study population are presented in Table 1. No differences were found for age, gravidity, parity, and time since last pregnancy (index pregnancy) between the two groups. fPE women had a significantly higher BMI compared to fHP-women both during LS and HS diet. Hip to waist ratio did not significantly differ between groups. Urinary sodium concentrations showed that the dietary compliance during LS and HS diet was excellent in both groups. No statistically significant differences in potassium and urea excretion were found between the groups reflecting an equal intake of potassium and proteins. Serum sodium did not differ between fHP en fPE during both LS and HS diet. No differences in PRA and aldosterone were found between the groups, and the change in PRA and aldosterone in response to low sodium intake was similar in both groups. All women were normotensive at the time of the screening visit and there were no significant differences in mean baseline 2-hour blood pressure and pulse pressure measured after LS diet and HS diet week [46]. Both fHP and fPE women demonstrated a significant increase in blood pressure in response to HS as compared to LS, but there were no differences in blood pressure responses to diet (salt sensitivity) between the fHP and fPE women.

Table 1. Baseline characteristics

		History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	p-value
Age (years)		36 ± 5	36 ± 5	0.95
Gravidity		2.5 ± 1.3	2.6 ± 1.1	0.95
Parity		2.0 ± 0.7	2.2 ± 1.0	0.59
Elapsed time since index pregnancy (years)		4.2 ± 2.6	5.3 ± 3.0	0.24
Waist/Hip ratio		0.83 ± 0.04	0.84 ± 0.06	0.44
MAP (mmHg)	LS	81 ± 7	83 ± 8	0.38
	HS	85 ± 8	86 ± 9	0.71
PP (mmHg)	LS	43± 6	42 ± 5	0.72
	HS	44 ± 5	43 ± 5	0.62
HR (beats/min)	LS	67 ± 8	67 ± 9	0.95
	HS	67 ± 8	66 ± 10	0.64
BMI (kg/m^2)	LS	22.6 ± 2.6	25.3 ± 3.3	**0.01**
	HS	23.2 ± 2.7	25.9 ± 3.5	**0.02**
Urinary sodium (mmol/24h)	LS	39 ± 14	45 ± 23	0.33
	HS	221 ± 64	258 ± 86	0.15
Urinary potassium (mmol/24h)	LS	66 ± 21	76 ± 25	0.20
	HS	80 ± 34	73 ± 15	0.46
Urinary urea (mmol/24h)	LS	264 ± 91	306 ± 63	0.12
	HS	339 ± 89	340 ± 65	0.97
Serum sodium (mmol/l)	LS	140 ± 1.6	140 ± 1.9	0.36
	HS	142 ± 1.8	141 ± 2.4	0.31

		History of normotensive pregnancy (n = 18)	History of preeclamptic pregnancy (n = 18)	p-value
PRA (nmol ANG I·l^{-1}·h^{-1})	LS	0.80 (0.50-1.20)	0.85 (0.70-1.50)	0.50
	HS	0.20 (0.10-0.50)	0.20 (0.09-0.30)	0.58
Aldosterone (pmol/L)	LS	255 (204-395)	341 (214-477)	0.16
	HS	71 (29-93)	59 (35-96)	0.84
Aldosterone:PRA ratio	LS	331 (201-450)	456 (250-494)	0.38
	HS	224 (151-499	316 (181-517)	0.23
Change in PRA HS to LS (%)		225 (100-350)	325 (160-700)	0.18
Change in Aldosterone HS to LS (%)		320 (187-462)	436 (90-700)	0.48

Data are presented as means ± SD or as medians (25th-75th percentiles). LS: low sodium diet (<50mmol Na$^+$/24h), HS: high sodium diet (>200mmol Na$^+$/24h), MAP: mean arterial pressure, PP: pulse pressure, HR: heart rate, PRA: plasma renin activity, ANG: angiotensin.

Arterial stiffness

Arterial stiffness in fHP and fPE on LS en HS expressed as AIX and PWV is showed in Figure 1. No overall difference in AIx was found between groups and between diets. However, the GEE-analysis showed that LS intake was associated with a significant decrease in Aix in fHP women (p_{diet}*fHP = 0.016) while no effect of LS intake on AIx was observed in fPE women. As for AIx, no significant differences in AIx@75 were found between group and diet, but in the fHP women again a significant decrease in AIx@75 in response to LS diet (p_{diet}*fHP= 0.024) was observed, while there was no effect of LS intake on AIx@75 in fPE women. No effect of group (fHP or fPE) and diet (LS or HS) within groups was observed for PWV. Linear regression showed a positive relation between age and arterial stiffness under both low and high salt conditions (P<0.05 for age*AIx, age*AIx@75 and age*PWV). GEE analysis corrected for age showed that differences in age did not affect the adaptation of arterial stiffness in response to low salt.

Extracellular volume

ECV corrected for body surface area (ECV/BSA) in fHP and fPE on LS en HS is shown in Figure 2. ECV/BSA did no differ between groups, but we did observe an overall effect of diet (p_{diet} < 0.001). There was a significant decrease in ECV/BSA in response to LS diet within both groups (p_{diet}*fHP < 0.001 and p_{diet}*fPE= 0.01) without differences between the groups.

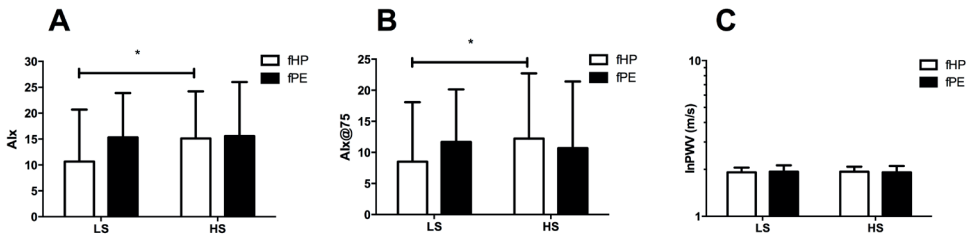

Figure 1. Arterial stiffniss during low and high sodium diet.

*Augmentation index (AIx, A), Augmentation index corrected for heart rate (AIx@75, B) and log pulse wave velocity (PWV, C) during low sodium (LS, white bars) and high sodium (HS, black bars) intake in women with a history of healthy pregnancy (fHP) and in formerly preeclamptic (fPE) women. Data are expressed as estimated marginal means ± standard error. *P < 0.05 by generalized estimating equation analysis.*

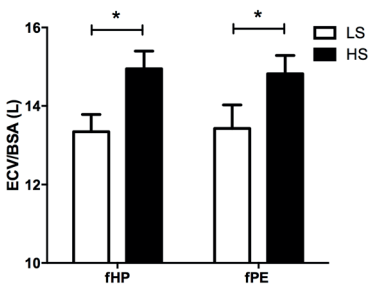

Figure 2. Extra cellular volume during low and high sodium diet.

*Extra cellular volume corrected for body surface area (ECV/BSA) during low sodium (LS, white bars) and high sodium (HS, black bars) intake in women with a history of healthy pregnancy (fHP) and in formerly preeclamptic (fPE) women. Data are expressed as estimated marginal means ± standard error. *P < 0.05; by generalized estimating equation analysis.*

Endothelial function markers

As markers for endothelial function, we measured plasma nitrate, RxNO and cGMP in fHP and fPE women (Figure 3). GEE analysis for nitrates showed no significant differences between groups while diet did affect plasma nitrates ($p_{diet} = 0.008$). Low salt intake significantly increased the nitrate concentration in both groups ($p_{diet*fHP} = 0.016$, $p_{diet*fPE} = 0.04$). We did not find differences in RxNO and cGMP concentrations between diet and groups but there was a large inter-individual variation of these parameters within our data.

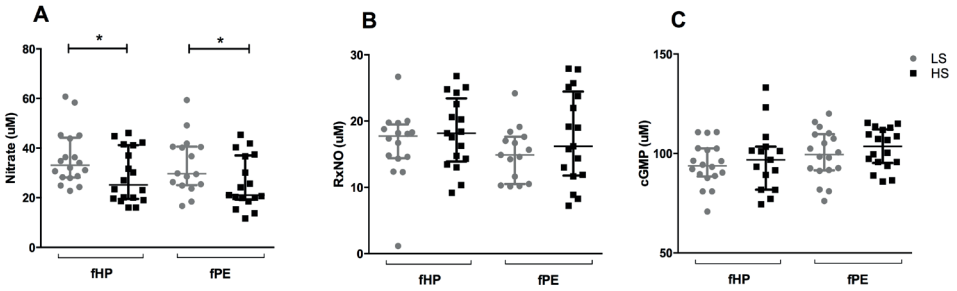

Figure 3. Endothelial function markers during low and high sodium diet.

*Nitrate (A), total total nitrosated species (RxNO, B) and cyclic GMP (cGMP, C) during low sodium (LS, grey circle) and high sodium (HS, black square) intake in women with a history of healthy pregnancy (fHP) and in formerly preeclamptic (fPE) women. Data are expressed as medians with interquartile ranges. *P < 0.05; by generalized estimating equation analysis.*

DISCUSSION

This is the first study that assessed arterial stiffness in formerly early-onset preeclamptic women without any comorbidity, under standarized low and high sodium conditions. We demonstrated that fPE women have an impaired ability to adapt their arterial stiffness (AIx and AIx@75) upon low sodium diet compared to fHP women. The non-adaptation of arterial stiffness could not be explained by differential responses between fHP and fPE women in blood pressure, extracellular volume expansion and plasma availability of nitrate, RxNO, and cGMP, or RAAS-activity. Increased arterial stiffness is known to be associated with the development of hypertension and cardiovascular diseases [5, 21, 28, 30]. An impaired ability to decrease AIx in response to low sodium in fPE women might be a first indication of an unfavorable vascular profile.

While both AIx and PWV were similar in fHP and fPE women on HS diet, LS diet reduced the AIx and AIx@75 in fHP-women but not in fPE-women. We are the first to show this effect of non-adaptation of arterial stiffness in a well-controlled setting, studying subjects at risk for premature vascular disease and healthy controls in absence of any comorbidity. LS diet normally reduces arterial stiffness; as was shown by a meta-analysis exploring the effect of dietary and nutritional interventions on arterial stiffness [32] and by a study in (postmenopausal) female hypertensive subjects [42]. Our finding of non-adaptation of arterial stiffness in response to LS in fPE thus suggests that fPE women lost the capability to adjust arterial stiffness upon LS intake.

Previous studies on arterial stiffness in fPE women were not performed under standardized dietary conditions and did not standardize for phase menstruation cycle. Assuming that sodium intake in the previous studies is in the range of average intake in the western diet, these studies are comparable with our HS condition under which we observed no differences in AIx and PWV between groups. Under this assumption, our results are in line with two other studies that showed no differences in AIx in fPE women compared to controls [24, 37]. However, other studies have reported increased arterial stiffness as measured by AIx [10, 27, 31, 35, 45, 53] as well as by PWV [31, 35, 45] in fPE women.

These conflicting findings probably result from differences in study design, the heterogeneity of the preeclamptic phenotype, and the presence of comorbidity (i.e. hypertension and increased BMI). The strength of our study is that we studied a well-characterized group of healthy fPE women under standardized dietary conditions matched to a control group. Therefore, we can conclude that fPE women without comorbidity do not differ in arterial stiffness compared to fHP women on a regular western diet.

Our finding of non-adaptation of arterial stiffness upon LS diet was only reflected in AIx and not in the PWV measurements. This might be explained by the different (age-related) vascular responses that these measures represent [29]. In general, the AIx is influenced by the resistance of the vessels and highly dependent on endothelial function. PWV is affected by structural changes such as narrowing and sclerosis of the vessels that occur during the late process of atherosclerosis. While PWV is the gold standard for measurement of arterial stiffness [47], AIx might be more accurate in detecting early stage vascular dysfunction based on sensitivity to detect functional instead of structural abnormalities [7, 48]. In our study all women were healthy and relatively young, and therefore one might not expect structural vascular abnormalities reflected by differences in PWV.

Increased arterial stiffness is a result of aging (changes in extracellular matrix composition) [22] and is associated with hypertension, diabetes mellitus, atherosclerosis and renal failure [33, 38]. We carefully excluded these factors from our study design by matching our fPE group with a control group of the same age and by the exclusion of women with co-morbidity. To overcome the influences of differences in sodium intake on arterial stiffness we had an excellent standardization of the diets as observed in the urinary sodium values. On both LS and HS serum sodium did not significantly differ between fHP en fPE but interestingly there was a slightly different response of serum sodium to the change in diet within groups (fHP LS vs HS $p<0.05$, while fPE LS vs HS ns; p-values earlier not shown). This slight difference might have influenced the non-adaptation of arterial stiffness in response to LS in the fPE group.

Other important determinants of arterial stiffness are the RAAS [14, 43], volume status [20] and endothelial function [39]. These determinants are also known to be affected by dietary sodium intake and could therefore be mechanisms underlying non-adaptation of arterial stiffness in response to sodium [9]. However, we did not detect any differences in circulating RAAS components between groups and we found that the systemic RAAS was adequately modulated by sodium intake in both groups as observed by a similar increase in PRA and aldosterone in response to LS in our study. Our ECV data show that both the fHP and fPE group are reducing their ECV upon LS to a similar extent. As expected, the volume reduction resulted in reduction in arterial stiffness in fHP women, illustrating the ability of healthy vessels to shift stiffness along their compliance curve in response to volume reduction. Since non-adaptation of arterial stiffness was observed in fPE women, we hypothesize that fPE women have stiffer vessels that already work at the upper end of their compliance having less adaptability upon extrinsic factors.

In respect to endothelial function, plasma nitrate, RxNO and cGMP did also not differ between groups, while both groups showed the expected increase in plasma nitrate on LS. Plasma nitrate, RxNO and cGMP are part of the L-arginine-NO-synthase pathway (nitrate as precursor

for NO [26], and cGMP as activated product of the NO-pathway), which play an important role in regulation of endothelial function (relaxation). Based on our findings we cannot conclude with certainty that endothelial function is not involved in the non-adaptation of arterial stiffness. A limitation of our study is that we did not assess flow-mediated dilatation (FMD), the gold standard to assess endothelial function. Previous work suggests that fPE women have an impaired FMD compared to controls [6, 16, 18, 31, 41, 53] and this might be a mechanism associated with non-adaptation of arterial stiffness under LS.

Future studies should investigate the mechanistic pathways behind the non-adaptation of arterial stiffness in fPE women and should include arterial stiffness measurements in combination with FMD. It would be of interest to characterize the structure and character of the vessel wall and endothelial surface layer (glycocalyx). The glycocalyx plays an important role in both sodium homeostasis and regulation of arterial stiffness. Under high salt conditions the endothelial sodium channels are upregulated resulting in an increased sodium influx in the endothelial cells, which leads to increased stiffness [23]. Defects in the glycocalyx might result in increased arterial stiffening under LS condition by increased access of sodium to the sodium channels of the endothelium and subsequently reduced NO release causing contracted smooth muscle cells and vasoconstriction [13, 23]. In addition, the question whether inability to adapt arterial stiffness in response to LS was pre-existing or induced by preeclampsia remains to be answered.

In conclusion, this study is the first to show an effect of non-adaptation of arterial stiffness in healthy fPE women in response to LS. We could not explain the non-adaptation by differences in blood pressure, plasma RAAS parameters, ECV, plasma nitrate and plasma NO availability. The exact underlying pathways involved in the non-adaptation in this fPE group remain therefore to be elucidated. However, independent of the underlying mechanisms, impaired adaptation of the arterial stiffness in response to LS might be a marker of subclinical vascular damage in fPE women without comorbidity. We propose that non-adaptation in response to LS is a first sign of unfavorable vascular alterations in fPE women, which might put them at risk to develop hypertension and cardiovascular disease.

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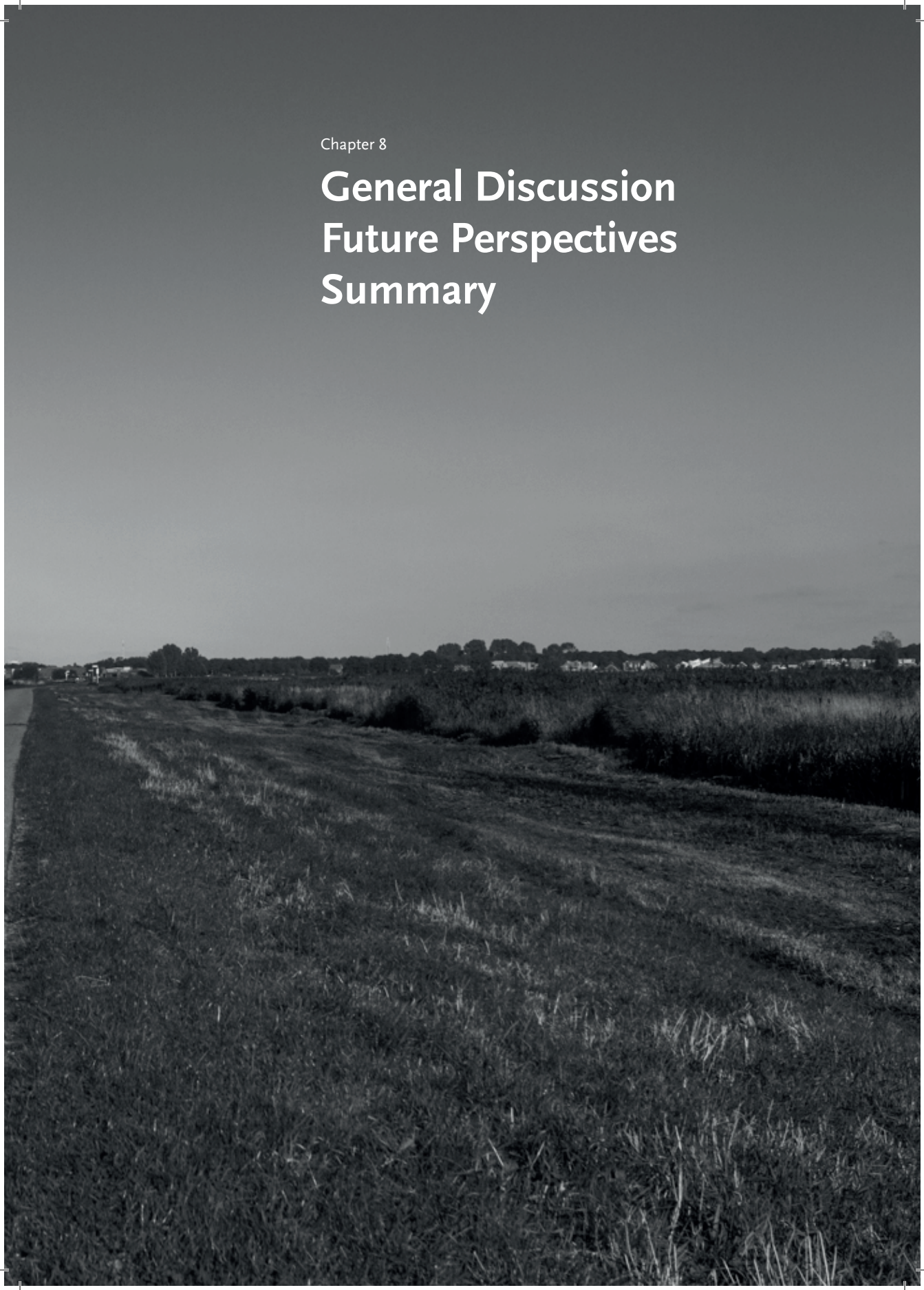
Chapter 8

General Discussion
Future Perspectives
Summary



GENERAL DISCUSSION

Preeclampsia is a syndrome affecting maternal and fetal health during gestation that also has an impact on the long-term health of women. Formerly preeclamptic women are more susceptible to cardiovascular and renal disease later in life as compared to women with a history of a healthy pregnancy. Whether it is preeclampsia per se or shared risk factors for preeclampsia and cardiovascular and renal disease inducing this increased risk is unknown. In addition, the mechanisms behind this increased susceptibility remain to be elucidated. Both questions were addressed in this thesis.

We hypothesized that increased angiotensin II (ang II) sensitivity and endothelial dysfunction might be involved. We first validated a rat model for preeclampsia for these specific features assumed to be involved in the postpartum increased risk for cardiovascular and renal disease (**Chapter 2**). Thereafter, we performed postpartum studies in formerly preeclamptic women and formerly experimental preeclamptic rats (**Chapter 3-7**). We aimed to assess whether disturbances seen during preeclampsia, i.e. altered renal hemodynamics, increased ang II sensitivity, and endothelial dysfunction, persisted postpartum in order to elucidate whether these mechanisms might be involved in the increased susceptibility to cardiovascular and renal disease (Figure 1). Elucidating these mechanisms will not only help to explain why formerly preeclamptic women are more susceptible for cardiovascular and renal disease, but will also be an important step towards structured follow-up and specific preventive treatment of these women.

The angiotensin II sensitivity and endothelial function in experimental preeclampsia

In this thesis, we used the low-dose endotoxin rat model for preeclampsia. This model is based on the increased inflammatory status seen in human preeclampsia and is pregnancy specific [1]. In this model, the two main preeclampsia-like features, hypertension and proteinuria, have been described extensively by our group and others [1-5]. Previous studies found glomerular inflammation [6] and increased activation of circulating monocytes and granulocytes [7, 8]. In addition, fetal and placental involvement have been shown [4, 5].

In **Chapter 2** we studied ang II sensitivity and endothelial function in pregnant rats with low-dose endotoxin induced preeclampsia [1], both characteristics of the multifactorial disease of preeclampsia [9-11]. In healthy pregnancy, sensitivity to ang II is decreased [12], while during preeclampsia, ang II sensitivity is increased [9, 10]. In addition, healthy pregnancy is characterized by an altered regulation of the endothelial-derived vasodilating factors leading to increased vasodilation, while in preeclampsia endothelial dysfunction occurs, leading to impaired vasodilation [11]. To assess ang II sensitivity and endothelial (dys)function, *ex-vivo* isotonic contraction experiments in the thoracic aorta were performed.

Similar to the human situation, decreased ang II sensitivity was seen during healthy pregnancy in the rat and sensitivity to ang II was increased during experimental preeclampsia. Interestingly, the experimental preeclamptic rats responded similarly to ang II as non-pregnant rats, suggesting maladaptation to pregnancy in experimental preeclampsia. In human pregnancy, the maladaption in ang II sensitivity seen in preeclampsia, is already present during the first trimester of

pregnancy [9]. Since endotoxin was infused on day 14 of rat pregnancy (i.e. second half of pregnancy) and ang II sensitivity was assessed at the end of gestation, it can be suggested that pregnancy-specific adaptations are reversed in experimental preeclampsia. Increased ang II sensitivity may cause generalized vasoconstriction in the vascular system and therefore contribute to the development of hypertension seen in preeclampsia [13]. Moreover, the increased ang II sensitivity in this model might also contribute to the development of other preeclampsia-like features since blocking the ang II type I receptor (AT1-R) ameliorated hypertension, proteinuria, and fetal outcome in this model [5].

Although several mechanisms have been proposed to be involved in the changes in ang II sensitivity, in **Chapter 2** we mainly focused on the role of the AT1-R and the ang II type II receptor (AT2-R) in the ang II sensitivity. The AT1-R mediated vascular constriction and AT2-R mediated vascular relaxation were increased in experimental preeclampsia as compared to healthy pregnancy and an increased AT1-R/AT2-R mRNA balance in the thoracic aorta of the experimental preeclamptic rat was found. The recently suggested phenotypic change of the AT2-R towards a constrictor receptor during preeclampsia, thereby contributing to the enhanced ang II responsiveness in preeclampsia [10], seemed not to be present in our animal model for preeclampsia. We found increased AT2-R mediated vasodilation in experimental preeclampsia as compared to the healthy pregnant rats. Since experimental preeclampsia was induced in the second half of pregnancy and ang II sensitivity was studied at day 19 of gestation, studying *in-vivo* and *ex-vivo* responses of ang II via the AT2-R, at different time points during gestation, is of interest. In rat the uterus [14, 15] and the rat kidney [16] for example, it has been shown that ang 1-7, ACE2, and AT2-R expression varied throughout gestation. Therefore, it can be suggested that in order to adapt to the pregnancy-induced maternal hemodynamic changes, the magnitude and site of expression of the ang II receptors varies during gestation, thereby affecting ang II responses and receptor function. During preeclampsia this ingenious RAAS regulation might then be disturbed via for instance endothelial dysfunction, secreted factors from the stressed placenta directly influencing the RAAS (i.e. soluble fms-like tyrosine kinase-1 (sFlt1)) or via AT1-R auto-antibodies (AT1-AA) influencing receptor activity and expression.

In **Chapter 2**, in addition to the role of the RAAS, the role of endothelial (dys)function on vascular tone was assessed during pregnancy and in experimental preeclampsia. The increased role of NO in the pregnancy-induced vasodilation [17] was not seen in our study. This coincides with the lack of differences in eNOS and iNOS mRNA expression found in our study. The absence of the increased role for NO in vasodilation suggests that in these rats, NO is of less importance in the pregnancy-induced vasodilation. To study this in more detail, NO metabolites (nitrate, nitrate) and NO activity (cGMP) should be assessed in future studies. In addition, the role of NO in the vasodilation of mesenteric arteries can be studied in order to elucidate whether pregnancy-induced changes are vascular bed dependent and is different in restrictive vessels. Whereas the role of NO was not affected by pregnancy, we did observe involvement of the prostanoids and EDHF during pregnancy as we found an increased role for vasodilating prostaglandins and a decreased role for EDHF. In experimental preeclampsia, these pregnancy-specific adaptations of the different endothelial-derived factors were absent. The experimental preeclamptic rats showed similar

vasodilating responses as the non-pregnant rats. This emphasizes again a reversal of the vascular adaptations to pregnancy during experimental preeclampsia. To elucidate this in more detail, in futures studies it would be useful to assess endothelial-dependent vasodilation at different time points during gestation.

Overall, in **Chapter 2**, we have shown that in the endotoxin model for preeclampsia changes in the RAAS and endothelial function occur. These changes, increased ang II sensitivity and endothelial dysfunction, are also seen in human preeclampsia, suggesting that this model for preeclampsia resembles the human situation in these characteristics. We did not study the mechanisms by which increased ang II sensitivity and endothelial dysfunction were induced. However, it may be suggested that the inflammatory response, induced by the low-dose endotoxin infusion [18], may be involved since inflammatory responses are associated with endothelial cell activation [19, 20], potentially leading to endothelial dysfunction, and ang II mediated inflammatory responses have been described [21]. We were not able to detect increased sFlt1 levels in this model (unpublished data), however, the exact role of other angiogenic factors, i.e. placental growth factor, vascular endothelial growth factor (VEGF), or soluble endoglin, need to be examined. For example, VEGF deficiency might play an important role in the development of endothelial dysfunction since VEGF is important in maintaining endothelial health [22]. Since ang II sensitivity and endothelial dysfunction have been shown to be present, this animal model is appropriate to specifically study these impairments postpartum in order to elucidate the role of preeclampsia on the postpartum endothelial function and ang II sensitivity.

Renal function, RAAS, and cardiovascular system after preeclampsia

It has been known for some time now, that formerly preeclamptic women are more prone to develop end-stage renal disease later in life [23-25]. In **Chapter 3** we described potential mechanisms involved in this increased renal risk. We reviewed several studies that have investigated renal function, renal hemodynamics, and the RAAS in women after a pregnancy complicated by preeclampsia. These studies suggest (subtle) persistent impairments. However, since most studies were performed early after pregnancy, it is unknown whether these (sub)clinical impairments are still present, or even worsen, during follow-up at middle age. In addition, the mechanisms involved in the increased long-term risk for cardiovascular and renal disease need to be elucidated in order to make the step towards prevention.

Therefore, we first longitudinally assessed renal function and renal function decline in women with a history of a hypertensive disorder during pregnancy (**Chapter 4**). Renal function data from women with a history of a (hypertensive) pregnancy collected in the PREVEND (Prevention of REnal and Vascular ENdstage Disease) study, a general population based prospective observational cohort study, were used. In addition, renal function parameters 10-years postpartum were compared in a sub-cohort of women following a healthy pregnancy, following pregnancy induced hypertension (PIH) and following preeclampsia and/or HELLP-syndrome (**Chapter 4**) to study whether severity of the hypertensive disorder was associated with more prominent effects on renal function. Next, we tested the hypothesis that following preeclampsia, (renal) hemodynamic changes (including

endothelial function) and changes in the RAAS occur. Such changes may be involved in the increased renal risk following an early-onset preeclamptic pregnancy (**Chapter 5-7**; the RETAP (REsponse To Angiotensin II in formerly Preeclamptic women) study).

To elucidate the mechanisms involved in the increased long-term renal risk following preeclampsia, it is of importance to discriminate between the role of pre-pregnancy risk factors and preeclampsia-induced factors, to distinguish whether preeclampsia itself or common pre-pregnancy risk factors are involved in the postpartum susceptibility to renal disease after preeclampsia. The optimal study design would obviously require pre-pregnancy assessment, but his is extremely difficult to realize in clinical practice. As an alternative, studying formerly preeclamptic women in the absence of cardiovascular risk factors could also shed light on the role of preeclampsia per se. In the RETAP study, we therefore carefully selected formerly early-onset preeclamptic women who were otherwise healthy, i.e. normotensive with a healthy body weight and without cardiovascular or renal diseases or current drug use (**Chapter 5, 6, and 7**). To study postpartum effects of preeclampsia in a fully clean model, animal studies are more practical; therefore, the endotoxin model for preeclampsia studied in **Chapter 2** was also used to study the effects of experimental preeclampsia on the postpartum RAAS (**Chapter 6**). Since in this model increased ang II sensitivity and endothelial dysfunction was found, it is a suitable model to study these particular impairments postpartum following experimental preeclampsia. In addition, no hypertension or proteinuria was found postpartum in our rats following experimental preeclampsia, showing that indeed healthy rats were studied.

In the next section, we will first elaborate on longitudinal renal function data collected during the PREVEND study in women following a hypertensive disorder during pregnancy (**Chapter 4**). Thereafter, the mechanisms suggested to be involved in the increased renal risk following preeclampsia that were studied in the RETAP study will be discussed (**Chapter 5-7**).

Renal function decline following a hypertensive disorder during pregnancy

Since formerly preeclamptic women exhibit an increased renal risk postpartum on the long-run, it is of interest to longitudinally assess renal function for an extended period of time. So far, only one study intended to investigate long-term renal function following preeclampsia. This cross-sectional small study suggested that age-dependent renal function decline was not affected by a history of preeclampsia [26]. In **Chapter 4** we presented unique longitudinal data from the PREVEND study. We are the first to show an accelerated renal function decline (assessed by estimated glomerular filtration rate (eGFR)) in women after a self-reported history of a hypertensive disorder during pregnancy. In addition, these women had slightly, but significantly lower eGFR as compared to controls, within the normal range. Both the steeper decline in renal function and the lower eGFR fit in the observation that these former hypertensive women were more susceptible to develop chronic kidney disease (CKD). However, as shown previously, the absolute risk for renal disease following preeclampsia is relatively low [23-25]. Also, the observed abnormalities in renal function after preeclampsia were subtle. Although our study highlights the importance of longitudinal follow-up, it remains questionable whether it is of additive value to assess renal function (i.e. eGFR) during follow-up in all women

following a hypertensive disorder of pregnancy. Importantly, formerly hypertensive women exhibited higher blood pressure values, lower renal function, and were using more anti-hypertensive drugs as compared to healthy controls, indicating that women with a history of hypertensive disorder of pregnancy carry a high-risk profile for future cardiovascular and renal disease. Whether this is a cause or a consequence of their hypertensive disorder during pregnancy and long-term increased health risk remains to be investigated in future studies.

As part of the women from the above mentioned PREVEND study delivered in one of the two hospitals in Groningen, we retrieved medical records from these women in order to obtain verified information on the severity of the hypertensive disorder during pregnancy (PIH, the HELLP syndrome, and preeclampsia). This allowed us to compare renal function data from women with PIH, HELLP or preeclampsia with healthy controls 10-years post-partum in a case-control set-up study (**Chapter 4**). The renal function data showed that women with former preeclampsia or HELLP syndrome had the highest risk for renal function impairment and CKD 10-years postpartum. It must be stated here that the prevalence of hypertensive disorders in the PREVEND cohort was higher than reported in other studies [27], possibly due to oversampling of subjects with albuminuria, by design of the PREVEND study. Consequently, our findings require confirmation in other cohorts.

If our data can be confirmed, this could provide a basis for recommendations to screen for renal abnormalities (i.e. plasma creatinine, eGFR, and/or albuminuria) at middle age (i.e. from postmenopausal onwards) in women with a history of preeclampsia or HELLP syndrome. In addition, in line with our national guideline for cardiovascular risk management (CVRM [28]), it should be strongly considered to establish a risk profile in all women with a history of (early-onset) preeclampsia/HELLP syndrome. However, before recommendations of standardized renal follow-up in women following a hypertensive disorder during pregnancy can be made, the exact time course at which, for example, renal function decline becomes evident, needs to be identified in long-term longitudinal studies. Furthermore, more mechanistic insight is necessary in order to interfere in this decline by the use of therapeutic interventions.

From preeclampsia to renal disease; a mechanistic role for an altered renal hemodynamic profile?

Several studies suggest persistent impairments in renal hemodynamics after preeclampsia [29-31], which might subsequently contribute to the increased renal risk in later life. However, this was mainly found in formerly preeclamptic women that were hypertensive at the time of study, and thus might be related to the hypertension per se. Therefore, these studies cannot discriminate between the role of comorbidities and the role of the former preeclampsia in inducing renal function impairments. Moreover, these prior studies did not standardize for sodium status or stage of menstrual cycle, both known to exert substantial effects of renal hemodynamics. In **Chapter 5**, we therefore assessed renal hemodynamics in healthy formerly pregnant women and healthy formerly early-onset preeclamptic women, during standardized sodium conditions (i.e. on low and high sodium intake), and during standardized stage of the menstrual cycle. We observed subtle long-term changes in renal hemodynamics, i.e. increased filtration fraction, in formerly preeclamptic women on both sodium intakes. Since healthy women were studied, this may suggest a role for preeclampsia itself

on postpartum renal hemodynamics. However, pre-pregnancy changes cannot be ruled out in the human situation as mentioned earlier.

The increase in filtration fraction found relatively short (1-10 years) after preeclampsia, might underlie the increased long-term renal risk in these women. Indeed, a high filtration fraction has been independently associated with progressive renal damage in CKD [32, 33]. Other potential mechanisms of renal function loss could be the higher blood pressure, and/or presence of additional cardiovascular risk factors, as these are generally associated with an increased risk for long-term renal function loss. As described previously and studied in **Chapter 4**, in formerly preeclamptic women the rate of renal function decline with aging is more pronounced than in control women. This could potentially lead to overt CKD on long-term, as supported by the observed higher prevalence of CKD in the formerly preeclamptic women in our case-control study. Ideally, one would therefore assess postpartum renal hemodynamics longitudinally, in order to elucidate the time-course of these changes in renal hemodynamics. In addition, although the changes observed 1-10 years postpartum were relatively subtle, these changes can become of clinical significance with further progress at a more advanced age.

Several mechanisms could potentially be involved in the increased filtration fraction found in formerly preeclamptic women. An increase in vasoconstriction of the efferent arteriole relative to the afferent arteriole is one of the renal hemodynamic changes leading to an increased filtration fraction [34]. The RAAS is a known pathway that could induce this particular pattern [34]. However, no differences in renal ang II responses or RAAS parameters were seen in the formerly preeclamptic women studied in **Chapter 5**. To assess the mechanisms underlying changes in filtration fraction with certainty and to verify the role of the vasotonus in the arterioles, micropuncture studies [35] in animals that suffered from experimental preeclampsia may gain more insight. Studying renal hemodynamics in animals also allows standardizing conditions, i.e. sodium and RAAS status, body weight, and pre-pregnancy renal function. With regards to the structural impairments, animal studies are also ideal to assess whether glomerular lesions are present following experimental preeclampsia and whether they affect renal hemodynamics. In addition, mechanisms involved in inducing these glomerular lesions can be assessed, i.e. inflammation, imbalance in angiogenic factors or local RAAS activity.

From preeclampsia to renal disease; a mechanistic role for the renin-angiotensin aldosterone system?

As observed in **Chapter 2** and earlier mentioned studies, during preeclampsia ang II sensitivity is increased and therefore it can be suggested that increased ang II sensitivity persists postpartum. Postpartum increased ang II sensitivity might then contribute to the increased renal risk. This can for example be directly via renal vascular tone regulation, or indirectly via increasing systemic blood pressure. So far, three studies investigated ang II sensitivity in women with a history of PIH or preeclampsia [36-38]. In general, all three suggest an increased ang II sensitivity postpartum. However, co-morbidity, i.e. hypertension or overweight, was present and studies were not standardized for sodium status or menstrual phase, both factors known to play a role in the activity of the RAAS. Therefore, one cannot conclude that is was preeclampsia itself that induced this postpartum

increased ang II sensitivity.

In order to investigate whether postpartum RAAS alterations are present after preeclampsia, blood pressure response upon ang II infusion was studied (**Chapter 6**). Although subtle, our data suggested an increased blood pressure response upon ang II infusion postpartum in women after an early-onset preeclamptic pregnancy. To further substantiate between the effect of co-morbidity and a history of preeclampsia on the increased ang II sensitivity postpartum, blood pressure and kidney response upon ang II infusion were studied in an experimental rat model for preeclampsia (**Chapter 6**). In line with the human study, also rats did not show hypertension or proteinuria following a healthy pregnancy or experimental preeclampsia. And again, increased responsiveness upon ang II was found in formerly experimental preeclamptic rats. Together, our human and experimental data suggest persistent ang II sensitivity following (experimental) preeclampsia which subsequently might contribute to the increased renal risk. In addition, since healthy women and pre-pregnancy identical rats were studied, it can be hypothesized that preeclampsia itself induces this increased ang II sensitivity postpartum.

To assess the mechanisms behind the increased ang II sensitivity postpartum, the function and role of the aligned receptors for ang II is of interest. Since in our experimental work we have shown an increased AT1-R response during preeclampsia (**Chapter 2**) while a diminished AT2-R response was seen after preeclampsia (**Chapter 6**), it may not merely be a persistence of ang II sensitivity following preeclampsia, there also appears to be a transition in the balance between these receptors. In addition, the postpartum presence of AT1-AA (which we found in women following preeclampsia (**Chapter 6**)) might play a direct role in the increased ang II sensitivity or indirectly influences this receptor balance.

From preeclampsia to renal disease; a mechanistic role for altered arterial stiffness?

Increased arterial stiffness is an independent precursor of cardiovascular diseases [39, 40] and could be a candidate mechanism for the increased renal and cardiovascular risk in formerly preeclamptic women. Several studies have reported increased arterial stiffness in women with a history of preeclampsia [41-43]. However, these studies show inconsistencies and in the most recent studies no differences in arterial stiffness were reported. However, none of the studies were performed under standardized sodium conditions. Since a high sodium intake increases arterial stiffness [44] while a low sodium intake reduces arterial stiffness [45, 46] in **Chapter 7**, arterial stiffness was assessed in healthy formerly preeclamptic women under low and high sodium intake. Although baseline blood pressure and blood pressure responses to the change in sodium intake were comparable between the groups, formerly preeclamptic women did not adapt arterial stiffness during the change in sodium intake. This non-adaptation of arterial stiffness has not been described previously, neither in normotensive subjects [47] nor in hypertensive subjects [46]. In line with previous studies, although now under standardized conditions, i.e. a high sodium intake (corresponding to the normal Western diet), no differences were observed between formerly healthy women and formerly preeclamptic women. However, after challenging women with a low sodium diet, differences between the groups became apparent.

The mechanism behind this non-adaptation of arterial stiffness in formerly preeclamptic women needs to be elucidated. Several mechanisms have been suggested to be involved in arterial stiffness regulation. Aging increases arterial stiffness probably via changes in the extracellular matrix composition [48]. Also hypertension, diabetes mellitus, atherosclerosis, and renal failure increases arterial stiffness [49, 50]. Since in **Chapter 7** only women without comorbidity were studied and women were in the same age range, these mechanisms are not likely to have influenced our results. Both the RAAS [51, 52] and volume status [53] are important determinants of arterial stiffness. Our data presented in **Chapter 7** suggest that in our study, the RAAS was not mechanistically involved in the non-adaptation of arterial stiffness since no differences in systemic RAAS parameters were found between groups. Although low sodium reduced volume status in both groups to a similar extent, only formerly healthy pregnant women were capable in reducing arterial stiffness. We feel that in our study, sodium status may have been the factor determining arterial stiffness. Indeed, sodium status is suggested to regulate arterial stiffness [46, 54, 55] and we found sodium status to differently affect arterial stiffness. Future studies thus should aim on exploring the mechanism behind this regulation. For example, serum sodium response was slightly different between our groups studied and this might have influenced the non-adaptation of arterial stiffness in formerly preeclamptic women.

With increased arterial stiffness, signs of endothelial dysfunction might be expected [56]. The gold standard to assess endothelial function is by measuring the flow-mediated dilatation (FMD). Since previous studies found an impaired FMD in formerly preeclamptic women [41, 57, 58], endothelial dysfunction can be suggested as a mechanism behind the non-adaptation of arterial stiffness in formerly preeclamptic women. As in human preeclampsia it has been suggested that endothelial dysfunction is associated with decreased NO bioavailability [11, 59]. We hypothesized that postpartum increased arterial stiffness is partly caused by decreased NO availability or activity. However, assessment of nitrate and cyclic GMP, surrogates for NO production and activity, could not confirm this in our formerly preeclamptic women (**Chapter 7**). Whether other endothelial-dependent (i.e. prostanoids or EDHF) or endothelium-independent (i.e. smooth muscle cells) factors are involved deserves further research. In line with the suggestion of endothelial dysfunction following preeclampsia, animal work in our group, has shown subtle signs of disturbed endothelial-dependent relaxation in formerly preeclamptic rats (unpublished results). In the absence of increased blood pressure postpartum, the contribution of the vasoactive factors to relaxation of the thoracic aorta differed between formerly healthy pregnant rats and formerly preeclamptic rats, i.e. more NO and less EDHF for endothelial-dependent relaxation in formerly preeclamptic rats.

Final conclusion

Our studies in formerly preeclamptic women and (formerly) experimental preeclamptic rats in this thesis demonstrated long-term consequences of preeclampsia. Our studies point toward a role for preeclampsia per se on the postpartum impairments in women with a history of preeclampsia, thereby increasing the susceptibility of these women to develop cardiovascular and renal disease later in life. Our studies demonstrated that postpartum changes in renal hemodynamics, persistent increased ang II sensitivity, endothelial dysfunction, and arterial stiffness all provide leads for further research into the exact mechanisms behind this increased susceptibility (Figure 1). Using a history of preeclampsia as an early marker to identify young women at risk for premature cardiovascular and renal disease opens a window of opportunities for preventive treatment in this specific group of women, even in the absence of known cardiovascular risk factors.

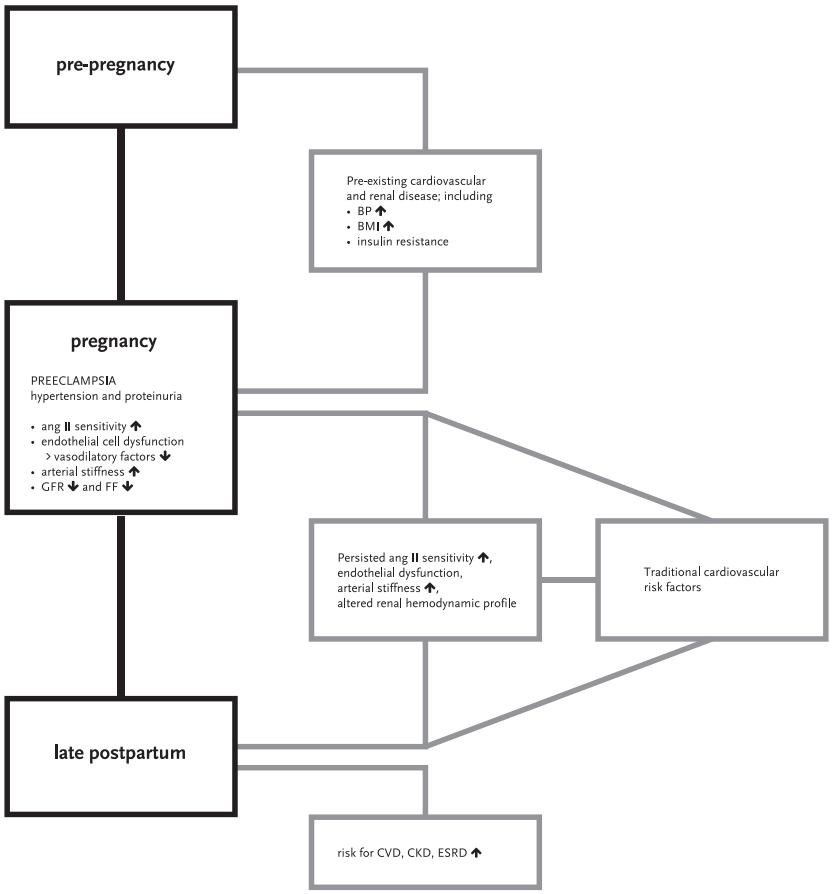


Figure 1. Simplified version adapted for this thesis of proposed and studied mechanisms involved in the long-term renal health risk following preeclampsia.

So far, it is unknown whether women who develop preeclampsia during pregnancy, exhibit prior to pregnancy an altered cardiovascular health status, i.e. pre-existing cardiovascular and renal disease risk factors. These pre-existing factors might increase a woman's risk to develop preeclampsia during pregnancy. Preeclampsia is clinically characterized by hypertension and proteinuria, with alterations in ang II sensitivity, endothelial function, renal function, and arterial stiffness involved. Our thesis has demonstrated that these specific alterations persist postpartum and we hypothesize that these persistent alterations increase the susceptibility of these women to develop renal disease late postpartum. In addition, traditional cardiovascular risk factors, possibly already present pre-pregnancy, might add to this increased long-term renal risk.

BP: blood pressure, BMI: body mass index, ang II: angiotensin II, GFR: glomerular filtration rate, FF: filtration fraction, CVD: cardiovascular diseases, CKD: chronic kidney disease, ESRD: end-stage renal disease

FUTURE PERSPECTIVES

Experimental preeclampsia; what can we learn from it and what should be the next step?

Our animal work has shown pregnancy specific adaptations of the vascular bed with preeclampsia leading to maladaptation. Changes in the contribution of endothelial derived vasoactive factors induced by pregnancy were seen, while during preeclampsia regulation of endothelial-derived vasodilatory factors was altered, suggesting endothelial dysfunction. Also, a pregnancy-specific decrease in ang II sensitivity was observed, which was absent during experimental preeclampsia. However, how experimental preeclampsia affects ang II sensitivity and the endothelial cells has still not completely been elucidated. Therefore, future studies should focus on the intriguing interaction between (experimental) preeclampsia and the vascular bed. By unraveling the exact mechanisms and pathways behind the changes in the vascular bed to pregnancy, via more experimental animal work, future studies can then be conducted focusing on protecting the vascular bed from these changes, and hence from preeclampsia.

Since preeclampsia is a multifactorial disease, with, as we have shown, the inflammatory system, endothelial dysfunction, renal function, and the RAAS involved, combining these pathways in a future animal model, for instance low-dose endotoxin infusion and RAAS over activity combined, might provide more insight into its multifactorial origin. Combining these pathways in a future animal model might hypothetically exaggerate preeclampsia-like features, i.e. hypertension and endothelial dysfunction, via inducing a common final pathway following these distinct but coinciding pathways. Hypothetically, when symptoms exaggerate, it can be stated that the pathways are either independently involved and/or reinforce each other.

Long-term consequences of preeclampsia; from a mechanistical point of view

Since we have shown persistent impairments in renal function, RAAS activity, and vascular function in women with a history of preeclampsia that hypothetically induce their susceptibility to renal disease later in life, more studies are warranted that elucidate the mechanisms involved in more detail.

From a renal perspective, the ultimate goal is to protect formerly preeclamptic women from long-term renal disease. Therefore, to gain more mechanistic insight into renal function decline and altered renal hemodynamics, micropuncture studies in animals following preeclampsia focusing in detail on the role of the glomerular pressure and glomerular lesions in the increased filtration fraction should be performed. It addition, longitudinal studies should be performed in order to investigate which women, i.e. women with a specific cardiovascular/renal risk profile or lifestyle, suffer the highest risk for renal function decline on the long-run and to test whether for example RAAS-blockade, protects against the suggested switch from hyperfiltration to hypofiltration that eventually leads to renal function decline.

With regards to the increased ang II sensitivity, the potential of RAAS blockade in reducing the long-term risk should be assessed in for example experimental preeclampsia. In addition, longitudinal ang II sensitivity studies in (experimental) preeclampsia during gestation (*ex-vivo*)

and postpartum (*in-vivo* and *ex-vivo*) are of interest to perform in order to test whether phenotypic changes in ang II receptors occur, to verify the time course of these possible changes, and to assess the mechanisms behind the differentially involved AT1-R and AT2-R in the ang II sensitivity during and after preeclampsia.

Although no sodium-sensitivity of blood pressure was observed in formerly preeclamptic women, low sodium diet distinguished formerly preeclamptic women from healthy women with respect to arterial stiffness. Therefore, the effect of sodium status on the endothelial function following (experimental) preeclampsia is of interest to study. To further substantiate whether the increased arterial stiffness under low sodium intake is caused by impairments in the endothelial-dependent or endothelial-independent vasodilation, flow mediated dilation (FMD) and glyceryl trinitrate-induced vasodilation assessments can be performed respectively. Also, since oxidative stress is associated with endothelial dysfunction and inflammation, it is of interest to study oxidative stress markers, especially in combination with a low and a high sodium diet. To further verify the role of oxidative stress, studying the effect of anti-oxidants on arterial stiffness can be suggested. For example, arterial stiffness and FMD can be assessed under different sodium conditions, in the presence and absence of anti-oxidant treatment.

In addition to longitudinal studies, women with a history of preeclampsia more than 10 or 20 years postpartum can be studied to assess whether the differences we found become more pronounced over-time. Since the menopause is of influence on women's cardiovascular risk, menopausal women specifically following preeclampsia are of interest to study. In addition, since pregnancy is suggested to be a stress-test for life, future studies should also include a group of women that has never been pregnant.

The ideal future perspective: evidence-based structured follow-up and preventive treatment in women with a history of preeclampsia

A history of preeclampsia serves as a risk marker for future premature cardiovascular and renal disease. Preeclampsia history provides an opportunity for early identification of young women at increased risk, even in the absence of classical cardiovascular risk factors. Postpartum follow-up of these women can therefore detect early subclinical damage and gives the opportunity for preventive treatment and interventions at a younger age than usual. This thesis gives insight in renal function after preeclampsia and gives insight into mechanisms involved. To further elucidate the exact renal risk, more prospective case-control studies preferably at a high age, or retrospective studies in large non-selected population based cohorts, i.e. the LifeLines study in which pre-pregnancy data are available, are warranted.

Next to elucidating the effectiveness of lifestyle interventions in these women, it is of importance to verify the most optimal timing to start these interventions, i.e. directly postpartum or several years after the preeclamptic event. Longitudinal follow-up studies are therefore necessary to clarify the time course of cardiovascular and renal impairments to subsequently be able to detect the time point at which an adverse cardiovascular profile can be identified. The American Heart Association (AHA) and the American Stroke Association (ASA), both guidelines on prevention

of cardiovascular diseases, have recently included a history of preeclampsia as a women-specific risk factor for cardiovascular diseases. Both the AHA and ASA have implemented follow-up and treatment of formerly preeclamptic women in their guidelines [60, 61]. In addition, the Netherlands implemented a guideline on cardiovascular risk management after reproductive disorders in women [62]. However, the recommendations on screening and treatment are based on a low level of evidence. Therefore, more insight into the mechanisms behind this increased risk is necessary, since there is currently no real evidence how and whom to screen and which drugs and interventions will be of real benefit.

Preventive treatment can include both lifestyle interventions and pharmacotherapy. Since overweight and the metabolic syndrome are linked with both preeclampsia and cardiovascular diseases, a healthy diet and exercising should always be a general advice. Considering the effect of high sodium on renal hemodynamics, blood pressure, and the role of an increased filtration fraction in long-term renal risk, advice on sodium restriction should be incorporated into the dietary advices. Since ang II seems mechanistically involved in these women´s increased risk, the effectiveness of pharmacotherapy directed towards RAAS-blockade deserves further investigation on effectiveness. Albuminuria and increased blood pressure, both endangering cardiovascular and renal health, can effectively be treated by RAAS-blockade. Whether this is beneficial and in which subcategory at what age this should be started, needs to be elucidated. In line with the suggested persistent endothelial dysfunction, increasing the bioavailability of nitric oxide or supplementation of anti-oxidants can be suggested as a preventive treatment.

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SUMMARY

Preeclampsia, a pregnancy specific disorder, complicates up to 8% of the pregnancies worldwide. In the Netherlands, this is approximately 2%. The exact pathophysiology is still unknown, it is, however, generally accepted that preeclampsia is a two-stage disease with the placenta being organ of origin. The first stage of preeclampsia is characterized by poor placentation and during the second stage the placenta secretes several factors, which subsequently induce the maternal response; clinically characterized by hypertension and proteinuria.

Apart from termination of pregnancy there is no treatment option to cure preeclampsia. For a long time, it has been thought that preeclampsia is a completely reversible syndrome. However, it has been shown that women with a history of preeclampsia have an increased risk to develop cardiovascular and renal disease later in life. Although several mechanisms have been suggested to be involved in this increased risk, the exact mechanisms need to be elucidated. Whether common constitutional risk factors for both preeclampsia and cardiovascular disease are involved or whether preeclampsia itself induced the increased this risk is currently still unknown.

To gain more insight into the mechanisms behind the increased postpartum risk for cardiovascular and renal disease, we first evaluated angiotensin II sensitivity and endothelial function in an excisting rat model for preeclampsia (**Chapter 2**). Then, **Chapter 3-7** describes long-term renal function after preeclampsia and after experimental preeclampsia, together with studies investigating suggested mechanisms behind the postpartum increased risk for cardiovascular and renal disease in women with a history of preeclampsia.

An animal model for preeclampsia; focus on angiotensin II sensitivity and endothelial function

Pregnancy is characterized by major maternal hemodynamic changes. Blood volume increases and systemic vascular resistance decreases. The renin-angiotensin aldosterone system is an important system involved in this volume and tonus regulation. Healthy pregnant women show decreased sensitivity for the actions of the vasoconstrictor angiotensin II (ang II). In contrast, preeclampsia is characterized by increased sensitivity for ang II, contributing to hypertension and proteinuria. In **Chapter 2** we showed that in the low-dose endotoxin rat model for preeclampsia both endothelial dysfunction and alterations in the renin-angiotensin system are present. Healthy pregnant and non-pregnant rats were compared with pregnant rats infused with a low-dose endotoxin. Isolated aortic rings were used to perform isotonic contraction measurements. Acetylcholine dose-response curves were used to study endothelial dysfunction and ang II dose-response curves were used to evaluate ang II sensitivity and the role of the ang II type I and type II receptor (AT1-R and AT2-R respectively). We observed an increased role for the vasodilator prostaglandin and ang II sensitivity was decreased in healthy pregnant rats. This was associated with decreased responses of the AT1-R and AT2-R. However, these pregnancy adaptations were absent in rats with experimental preeclampsia. Therefore, the lack of vascular adaptations to pregnancy may play a role in the development of hypertension in this model. Accordingly, the low-dose endotoxin rat model is therefore a suitable

model to study potential treatment options interfering with the endothelium and ang II and to unravel the mechanisms behind these changes. Moreover, this model can be used to assess ang II sensitivity following experimental preeclampsia to investigate whether increased ang II persists postpartum as a mechanism behind the increased risk for cardiovascular and renal disease in formerly preeclamptic women.

Renal function, RAAS, and cardiovascular system after preeclampsia

Chapter 3 reviews the current understanding of the association between preeclampsia and the subsequently increased risk of developing chronic kidney disease (CKD). This review first elaborates on the increased renal risk in formerly preeclamptic women and provides an overview of the literature focusing on microalbuminuria and renal hemodynamics in these women. Next, we commented on angiogenic factors and the renin-angiotensin aldosterone system as suggested mechanisms by which the risk of CKD is increased in formerly preeclamptic women. Levels of soluble fms-like tyrosine kinase-1 (sFtl1), the antiangiogenic factor involved in the pathogenesis of preeclampsia, is suggested to be elevated postpartum and via impairing the vascular health might contribute to the development of reno-vascular diseases in later life. In addition, the presence of auto-antibodies to the AT1-R in formerly preeclamptic women and persistent increased ang II sensitivity postpartum is discussed. Unfortunately, it remains still unknown whether the association between preeclampsia and the increased risk for renal diseases can be explained by deleterious effect of preeclampsia itself on the kidney or by underlying risk factors that make women more susceptible to both preeclampsia and renal disease.

Using data from the Pevention of REnal and Vascular ENd-stage Disease (PREVEND) study allowed us to study long-term renal function and renal function decline over time in women with a history of hypertensive disorder during pregnancy (**Chapter 4**). Women with a history of a self-reported hypertensive disorder during pregnancy and women without a history of a self-reported hypertensive disorder during pregnancy were identified. Data on estimated glomerular filtration rate, albuminuria, and the prevalence of CKD over a time-period of 14-years were collected. Next, detailed information on pregnancy history were collected from the women that delivered in the two local hospitals and a case-control study was performed. In this case-control study, renal function parameters 10-years postpartum were analyzed and compared between healthy controls, pregnancy-induced hypertension and formerly preeclamptic women in order to analyze whether the severity of the disease was related to the long-term renal function loss. We found that a history of a hypertensive disorder during pregnancy was associated with an increased decline in renal function over time. In addition, 10-years postpartum, estimated glomerular filtration rate was significantly reduced in formerly preeclamptic women as compared to healthy controls. Moreover, almost 20% of the formerly preeclamptic women fulfilled the definition of having CKD 10-years postpartum as compared to 8% of the control women. To gain more insight into the exact mechanism behind this increased risk for CKD, more studies focusing on renal hemodynamics in formerly preeclamptic women are warranted. In addition, we suggest regular clinical follow-up of women with a history of a hypertensive disorder during pregnancy to create a window of opportunities for preventive

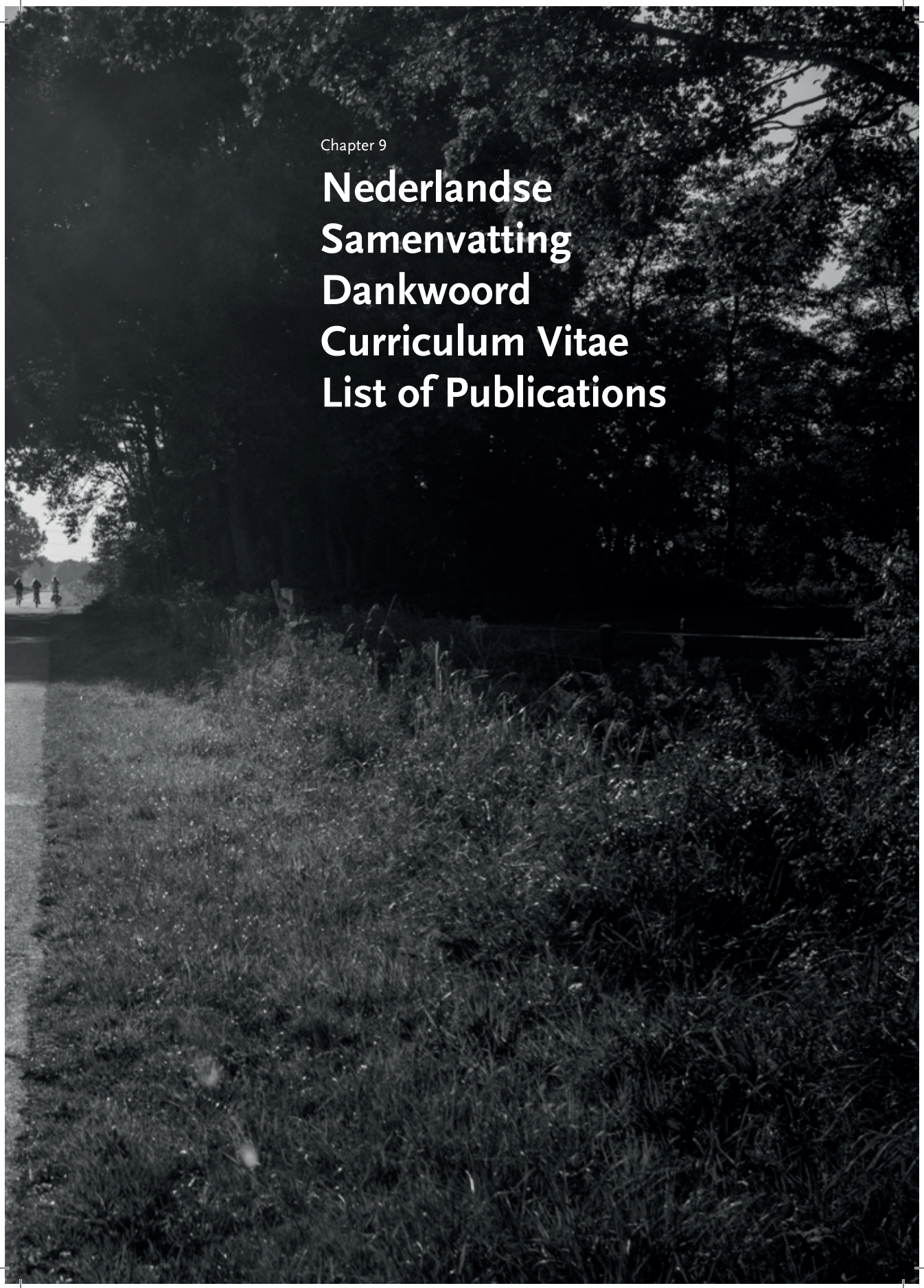
treatment. This follow-up should be based on evidence-based mechanisms found to be involved in women's increased susceptibility to renal disease, i.e. assessing renal function parameters (albuminuria, eGFR and/or creatinine).

Persistent impairments in the renal hemodynamics postpartum could be involved in the increased risk for end stage renal disease in women with a history of preeclampsia. In **Chapter 5** renal hemodynamics were studied in healthy formerly pregnant women and healthy formerly early-onset preeclamptic women 1-10 years postpartum. To measure glomerular filtration rate and effective renal plasma flow, the clearance of constantly infused radio-labeled tracers, ^{125}I-iothalamate and ^{131}I-Hippuran was assessed under standardized low sodium and high sodium conditions. In addition, ang II infusion was performed to investigate whether renal ang II sensitivity was increased, as an additional pathogenic mechanism behind the increased renal risk. We found that both the intake of high sodium and a history of preeclampsia increased filtration fraction. The renal response to ang II was not different between the groups. Thus, we demonstrated that preeclampsia has persistent effects on renal hemodynamics, even in the absence of co-morbidity. An elevated filtration fraction has been suggested to be involved in the development of hypertension and renal damage. Therefore, our results fit in the assumption that persistent disturbances in renal hemodynamics after preeclampsia, could be involved in the mechanism of the long-term increased risk for renal disease, independent of co-morbidity.

Persistent increased systemic ang II sensitivity postpartum is another suggested mechanism behind the increased risk for cardiovascular and renal disease in women with a history of preeclampsia. **Chapter 6** described a translational study investigating ang II sensitivity as the pressor response to ang II infusion, in healthy formerly pregnant women and healthy formerly early-onset preeclamptic women 1-10 years postpartum. Baseline blood pressure and blood pressure response upon ang II infusion were measured. Simultaneously, ang II sensitivity was studied both *in-vivo* and *ex-vivo* in never pregnant, formerly healthy pregnant, and formerly experimental preeclamptic rats. In both women and rats, baseline blood pressure was not affected after (experimental) preeclampsia. However, the pressor response to ang II was increased in formerly preeclamptic women and formerly experimental preeclamptic rats. Moreover, in response to ang II, only formerly preeclamptic rats had a significant increase in proteinuria. Since we only included healthy formerly preeclamptic women (without comorbidity) and all rats were healthy pre-pregnancy, our results support the hypothesis that preeclampsia itself plays an important role in the altered ang II sensitivity post-partum. This persistently increased ang II sensitivity might contribute to the increased cardiovascular and renal risk of formerly preeclamptic women in later life.

Arterial stiffness, described to be increased during preeclampsia, has also been suggested to be a mechanism behind the increased cardiovascular and renal risk in women with a history of preeclampsia. **Chapter 7** assessed arterial stiffness by measuring pulse wave analysis and pulse wave velocity under low and high sodium diet in formerly healthy pregnant women and formerly early-onset preeclamptic women in the absence of co-morbidity. We showed that formerly healthy pregnant women are able to modulate their arterial stiffness in response to a reduction of sodium intake. However, this modulation was absent in formerly preeclamptic women, independent of

blood pressure. To further elucidate the mechanisms, NO metabolites were measured as a reflection of endothelial (dys)function, and volume status response was analyzed. Differences in volume regulation or endothelial markers could not explain this non-modulation, so the mechanism behind this non-modulation remains to be elucidated.



Chapter 9

Nederlandse Samenvatting
Dankwoord
Curriculum Vitae
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NEDERLANDSE SAMENVATTING

Preeclampsie, in de volksmond bekend als zwangerschapsvergiftiging, komt wereldwijd in ongeveer 8%, en in Nederland in ongeveer 2% van de zwangerschappen voor. Preeclampsie is een zwangerschap-specifiek syndroom gekenmerkt door nieuw-ontstane hypertensie (hoge bloeddruk) en proteïnurie (eiwit verlies in de urine) in de tweede helft van de zwangerschap. Hoe preeclampsie ontstaat, is niet exact bekend. Wetenschappers erkennen preeclampsie als een syndroom dat zich ontwikkelt in twee stappen waarbij de placenta (moederkoek) een centrale rol speelt. De eerste stap kenmerkt zich door een verminderde aanleg van de placenta. Tijdens de tweede stap scheidt deze zieke placenta verschillende factoren uit in de moederlijke bloedbaan. Dit leidt in het lichaam van de moeder tot hypertensie en proteïnurie.

Preeclampsie is een gevaar voor de gezondheid van de moeder en het ongeboren kind, maar er bestaat helaas geen goede behandeling voor. De enige behandeling bestaat uit de geboorte van het kind, spontaan of door inleiden, of met een keizersnede. Lange tijd dacht men dat preeclampsie een voorbijgaand syndroom was en dat de moeder geheel genezen was na de bevalling. Echter, verschillende onderzoeken laten zien dat vrouwen die preeclampsie hebben doorgemaakt een verhoogd risico hebben op het ontwikkelen van hart-, vaat- en nierziekten later in het leven. Het mechanisme daarvan is onbekend. Evenmin is bekend of preeclampsie en hart- en vaatziekten het gevolg zijn van gemeenschappelijke aanlegfactoren, of dat preeclampsie zelf het risico op hart-, vaat- en nierziekten verhoogt.

Om meer inzicht te krijgen in het ontstaansmechanisme van preeclampsie en om mogelijke mechanismen achter het postpartum langtermijnrisico voor hart-, vaat- en nierziekten te onderzoeken, onderzoeken we eerst in een diermodel voor preeclampsie of er veranderingen optreden in het renine-angiotensine aldosteron systeem (RAAS) en in de endotheelfunctie. Daarna laten we de nierfunctie zien in vrouwen die preeclampsie hebben doorgemaakt 10 tot 15 jaar na de zwangerschap. Tevens beschrijven we studies (in bovenbeschreven diermodel en in de mens) waarin mogelijke mechanismen (waaronder het RAAS en endotheelfunctie) die bijdragen aan het verhoogde postpartum risico voor hart-, vaat- en nierziekten in vrouwen die preeclampsie hebben doorgemaakt zijn onderzocht.

Een diermodel voor preeclampsie; zijn de angiotensine II gevoeligheid en endotheelfunctie veranderd?

Zwangerschap kenmerkt zich door grote veranderingen in de hemodynamiek van de moeder. Het bloedvolume neemt toe en de weerstand van het systeemvaatbed neemt af. Het RAAS is een belangrijk systeem dat betrokken is bij zowel de volume regulatie als bij de regulatie van de vaattonus. Gezonde zwangere vrouwen hebben een verminderde gevoeligheid voor het vasoconstrictieve hormoon angiotensine II (ang II), de belangrijkste component van het RAAS. Bij vrouwen met preeclampsie daarentegen, heeft het vaatbed een verhoogde gevoeligheid voor ang II, hetgeen kan bijdragen aan de hypertensie en proteïnurie in preeclampsie. In **Hoofdstuk 2** onderzoeken we een

experimenteel model voor preeclampsie in de rat – het endotoxine model – om na te gaan of dit model endotheel dysfunctie met veranderingen in het RAAS vertoont, zoals bij de mens. In deze studie vergeleken we gezonde zwangere en niet zwangere ratten met zwangere ratten geïnfundeerd met endotoxine. Zwangere ratten geïnfundeerd met endotoxine ontwikkelen een ziektebeeld dat veel lijkt op preeclampsie, dat wil zeggen; ze ontwikkelen hypertensie en proteïnurie. De ang II gevoeligheid en endotheelfunctie werden onderzocht in geïsoleerde aorta ringetjes van deze groepen ratten, door middel van isotone contractie experimenten met concentratie-response curves met acetylcholine (endotheel functie) en ang II. Bij de gezonde zwangere ratten was er een toename van de vaatverwijdende prostaglandines en een verminderde response op ang II. Deze zwangerschap-specifieke aanpassingen zagen we niet in ons preeclampsie model. Op basis hiervan gaan we er vanuit dat verminderde vasculaire adaptatie aan de zwangerschap een rol speelt in de ontwikkeling van hypertensie in dit diermodel voor preeclampsie. Bovendien tonen deze resultaten dat het endotoxine preeclampsie model een geschikt model is om behandelingsopties te onderzoeken die invloed hebben op het endotheel en de verhoogde ang II gevoeligheid. Ook is het een geschikt model om het mechanisme van de verhoogde ang II gevoeligheid tijdens preeclampsie verder te onderzoeken, en om na te gaan of de verhoogde ang II gevoeligheid aanwezig blijft na afloop van de zwangerschap, als mogelijk mechanisme voor het verhoogde langetermijnrisico op hart-, vaat- en nierziekten na preeclampsie.

De nierfunctie, het RAAS en het cardiovasculaire systeem na preeclampsie

Hoofdstuk 3 geeft een overzicht van de huidige inzichten in de relatie tussen preeclampsie en het daarmee samenhangende verhoogde risico op chronische nierziekten. Het beschrijft eerst het toegenomen renale risico in vrouwen die preeclampsie hebben doorgemaakt en geeft een overzicht van de literatuur betreffende microalbuminurie en renale hemodynamiek in deze vrouwen. Vervolgens bespreken we de angiogene factoren en het RAAS als mogelijke mechanismen achter het verhoogde risico op chronische nierziekten na preeclampsie. De anti-angiogene factor *soluble fms-like tyrosine kinase-1* (sFlt1; een receptor voor *vascular endothelial growth factor* (VEGF)) is verhoogd in preeclampsie en speelt mogelijk een rol bij de ontwikkeling van preeclampsie. Verhoogde sFlt1 levels zorgen voor endotheel dysfunctie in het vaatbed, waardoor het mogelijk bijdraagt aan de ontwikkeling van hart-, vaat- en nierziekten later in het leven. Ook bespreken we de aanwezigheid van auto-antilichamen gericht tegen de ang II type I receptor in vrouwen die preeclampsie hebben doorgemaakt en bespreken we de blijvend verhoogde ang II gevoeligheid postpartum. Helaas weten we nog steeds niet of de relatie tussen preeclampsie en het verhoogde postpartum risico op hart-, vaat- en nierziekten verklaard wordt door de schadelijke effecten van preeclampsie zelf op de nieren, of dat onderliggende risicofactoren, zoals hoge bloeddruk, insuline resistentie en overgewicht, die voor de zwangerschap al aanwezig waren, een rol spelen bij zowel preeclampsie als nierziekten, deze associatie verklaren.

Door gebruik te maken van de gegevens van de PREVEND studie (*Prevention of Renal and Vascular End-Stage Disease*), waren we in staat om de langetermijnnierfunctie en nierfunctie achteruitgang te onderzoeken over een langere periode in vrouwen die wel of geen hypertensieve aandoening (zwangerschapshypertensie of preeclampsie) tijdens de zwangerschap hadden doorgemaakt (**Hoofdstuk 4**). Uit de PREVEND studie zijn vrouwen geselecteerd met en zonder een (zelf-gerapporteerde) voorgeschiedenis van een hypertensieve aandoening tijdens de zwangerschap. Van deze vrouwen hebben we de gegevens van de geschatte glomerulaire filtratie snelheid, eiwitverlies (albuminurie) en het vóórkomen van chronische nierziekten over een tijdsperiode van 14 jaar verzameld, en de nierfunctie en albuminurie tussen de twee groepen vergeleken. Wij zagen gedurende follow-up een versnelde achteruitgang in de nierfunctie in vrouwen die een hypertensieve aandoening tijdens de zwangerschap gerapporteerd hadden.

Om betere gegevens te verkrijgen over de zwangerschappen, hebben we voor de vrouwen uit de PREVEND studie die waren bevallen in de twee grote ziekenhuizen uit de stad Groningen, gedetailleerde zwangerschapsgegevens verzameld. Daarmee is een case-control studie uitgevoerd, die verschillende nierfunctie parameters 10-jaar postpartum tussen gezonde controles, vrouwen met zwangerschapshypertensie en vrouwen met preeclampsie in de voorgeschiedenis vergelijkt. Hiermee hebben we inzicht gekregen in de relatie tussen de ernst van de hypertensieve zwangerschapsaandoening, en het langetermijnrisico op nierschade. De geschatte glomerulaire filtratie snelheid was significant lager in vrouwen die preeclampsie hadden doorgemaakt vergeleken met de gezonde controles 10 jaar postpartum. Tien jaar postpartum voldeed ongeveer 20% van de vrouwen die preeclampsie had doorgemaakt aan de definitie van chronische nierziekte vergeleken met 8% van de gezonde controle vrouwen. Deze resultaten rechtvaardigen vervolgonderzoek naar de mogelijkheden van reguliere klinische follow-up van vrouwen die een hypertensieve aandoening hebben doorgemaakt tijdens de zwangerschap om zo hopelijk in de toekomst preventieve interventies en een geschikte behandeling aan te kunnen bieden.

Om meer inzicht te krijgen in het exacte mechanisme achter de versnelde achteruitgang in nierfunctie, zijn meer gegevens nodig over de renale hemodynamiek in vrouwen die preeclampsie hebben doorgemaakt. Blijvende verslechtering van de renale hemodynamiek postpartum is een van de mogelijke mechanismen achter het toegenomen risico op eindstadium nierfalen in vrouwen die preeclampsie hebben doorgemaakt. In **Hoofdstuk 5** is de renale hemodynamiek onderzocht in gezonde ex-zwangere vrouwen en gezonde vrouwen die *early-onset* preeclampsie (ontwikkelen van preeclampsie voor de 34e zwangerschapsweek) hebben doorgemaakt 1-10 jaar postpartum. Wij hebben gekozen om alleen gezonde vrouwen te includeren, dus vrouwen zonder hypertensie, overgewicht of insuline resistentie, om zo het effect van co-morbiditeit op onze resultaten te beperken. Om de exacte glomerulaire filtratiesnelheid en effectieve renale plasma doorstroming te bepalen, is de klaring van de continue geïnfundeerde radioactief gelabelde tracers, ^{125}I-iothalamaat en ^{131}I-Hippuran bepaald. Dit is in alle proefpersonen tweemaal gedaan, namelijk tijdens een laag natrium dieet en tijdens een hoog natrium dieet, omdat natrium een belangrijke modulator is van zowel het RAAS als van de renale hemodynamiek. Dit natriumdieet werd verkregen door de proefpersonen een laag en hoog zoutdieet voor te schrijven. De renale hemodynamiek werd

bovendien gemeten tijdens infusie van ang II om te onderzoeken of de renale ang II gevoeligheid is toegenomen als een mogelijk mechanisme voor het verhoogde renale risico na preeclampsie, maar deze bleek niet verschillend tussen de groepen. Wel vonden we dat zowel een inname van een hoog natrium als het hebben doorgemaakt van preeclampsie leidde tot een hogere filtratie fractie, dat wil zeggen dat vrouwen 1-10 jaar na preeclampsie hyperfiltreren. Deze resultaten laten dus zien dat in de afwezigheid van bovengenoemde co-morbiditeit, preeclampsie blijvende gevolgen heeft op de renale hemodynamiek. Omdat uit andere studies bekend is dat een verhoogde filtratie fractie kan bijdragen aan nierschade en hypertensie op de lange termijn, zou de toegenomen filtratie fractie bij vrouwen die preeclampsie hebben doorgemaakt mogelijk betrokken kunnen zijn bij de ontwikkeling van hypertensie en nierschade op de lange termijn bij deze vrouwen. Onze resultaten ondersteunen de veronderstelling dat een eerder doorgemaakte preeclampsie zelf een oorzakelijke factor zou kunnen zijn bij het verhoogde langetermijnrisico op nierschade, los van co-morbiditeit.

Blijvende verhoogde ang II gevoeligheid postpartum is een ander mogelijk mechanisme voor het verhoogde risico op hart-, vaat- en nierziekten na preeclampsie. In **Hoofdstuk 6** onderzochten we daarom in een humane- en dierstudie de ang II gevoeligheid van het vaatbed en de bloeddruk, in gezonde ex-zwangeren en in gezonde vrouwen die preeclampsie hebben doorgemaakt 1-10 jaar geleden, en in een ratmodel voor preeclampsie. Zowel de vrouwen als de ratten hadden een normale bloeddruk postpartum en waren verder gezond. In de vrouwen die een preeclampsie hadden doorgemaakt, was echter de bloedrukrespons op ang II infusie sterker dan in de controles. In de ratten die experimentele preeclampsie hadden doorgemaakt, was dit ook het geval. In de ratten die preeclampsie hadden doorgemaakt was er bovendien een significante toename in proteïnurie tijdens ang II infusie. Omdat onze vrouwen geen co-morbiditeit hadden en onze ratten gezond waren, ondersteunen onze resultaten de hypothese dat preeclampsie zelf een belangrijke rol speelt in de postpartum verhoogde ang II gevoeligheid, in zowel het systeemvaatbed als in de nier. Deze blijvende verhoogde ang II gevoeligheid kan bijdragen aan het verhoogde risico op hart-, vaat- en nierziekten in het latere leven in vrouwen na het doormaken van preeclampsie.

Arteriële vaatstijfheid, verhoogd tijdens preeclampsie, is ook een mogelijk mechanisme achter het verhoogde risico op hart-, vaat- en nierziekten na preeclampsie. In **Hoofdstuk 7** hebben we de vaatstijfheid bepaald met behulp van de *pulse wave analysis* en *pulse wave velocity* onder laag en hoog natrium in vrouwen na een gezonde zwangerschap en in gezonde vrouwen die preeclampsie hebben doorgemaakt 1-10 jaar postpartum en wederom in de afwezigheid van co-morbiditeit. Wij hebben laten zien dat gezonde ex-zwangere vrouwen in staat zijn de arteriële vaatstijfheid te moduleren op natriuminname. Deze modulatie was echter afwezig in vrouwen die preeclampsie hadden doorgemaakt, onafhankelijk van bloeddruk verschillen. De afwezigheid van vaatstijfheid modulatie zou een gevolg kunnen zijn van endotheel dysfunctie of van een afwijkende volume regulatie. Daarom hebben we stikstof metabolieten in het plasma gemeten als afspiegeling van endotheel (dys)functie en is de respons van de volume status op natriuminname bepaald. Verschillen in volume regulatie in respons op de natrium inname kon de gevonden non-modulatie niet verklaren. Ook zijn er geen verschillen gevonden in stikstofmetabolieten, die de endotheel functie weerspiegelen.

Daarom moet het mechanisme achter deze afwezige vaatstijfheid modulatie in vrouwen die preeclampsie hebben doorgemaakt nog opgehelderd worden.

Conclusie

De studies beschreven in dit proefschrift, uitgevoerd in vrouwen na preeclampsie en in ratten na experimentele preeclampsie, waren opgezet om mogelijke mechanismen achter het langetermijnrisico op hart-, vaat- en nierziekten in vrouwen na preeclampsie te onderzoeken. Wij hebben laten zien dat postpartum veranderingen in renale hemodynamiek, ang II gevoeligheid, endotheel functie en vaatstijfheid, betrokken kunnen zijn bij het verhoogde risico op hart-, vaat- en nierziekten in vrouwen die preeclampsie hebben doorgemaakt. Onze resultaten wijzen er bovendien op dat preeclampsie zelf een rol zou kunnen spelen in deze verhoogde kwetsbaarheid.

Door een voorgeschiedenis van preeclampsie te gebruiken als vroege risicofactor kunnen jonge vrouwen geïdentificeerd worden die kwetsbaar zijn voor het ontwikkelen van hart-, vaat- en nierziekten. Hoewel het noodzakelijk is meer onderzoek uit te voeren naar de door ons gevonden potentiële mechanismen achter dit verhoogde risico, zouden deze veranderingen aangrijpingspunten kunnen zijn voor preventie- en behandelingsstrategieën in deze specifieke groep jonge vrouwen, ook in de afwezigheid van bekende cardiovasculaire risicofactoren.

DANKWOORD

In 2006 maakte ik mijn eerste stappen in de wereld van het wetenschappelijk onderzoek. In de jaren daarna via verschillende cursussen van de JSM gegrepen door deze wereld, het enthousiasme van de mensen om mij heen en de vraagstukken die opgelost moeten worden. Vervolgens een bliksemstart tijdens mijn wetenschappelijke stage waarna een jaar onderzoeks- en levenservaring op doen in Australië. Bij terugkomst warm ontvangen in een geweldig team van begeleiders en onderzoekers waarmee ik met heel veel plezier heb samengewerkt om mijn MD/PhD-traject met succes af te ronden.

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Anne Marijn

CURRICULUM VITAE

Anne Marijn van der Graaf werd geboren op 5 mei 1987 te Oosterwolde. Op 1-jarige leeftijd verhuisde zij naar Appelscha. Zij bezocht het Stellingwerf College te Oosterwolde en behaalde daar in 2005 haar eindexamen VWO. Hierna studeerde zij één jaar Farmacie aan de Rijksuniversiteit te Groningen waarna zij werd ingeloot voor de studie Geneeskunde aan deze universiteit. Zij behaalde via de Junior Scientific Masterclass (JSM) haar Bachelor of Honours in 2009 waarna zij haar wetenschappelijke stage volgde onder leiding van dr. G.G. Zeeman bij de afdeling Obstetrie en Gynaecologie van het Universitair Medisch Centrum Groningen (UMCG). Via dr. Zeeman kwam zij in contact met prof. dr. G. Dekker in Adelaide (Australië) waar zij in 2010 een jaar onderzoek heeft gedaan bij de afdeling Obstetrie in het Lyell McEwen Hospital. Vervolgens werd zij toegelaten tot het MD/PhD-traject van de JSM. Onder leiding van prof. dr. G.J. Navis, prof. dr. S.A. Scherjon, dr. M.M.Faas en dr. A.T. Lely deed zij promotieonderzoek naar mechanismen die mogelijk betrokken zijn bij het verhoogde risico op nierziekten na het doormaken van preeclampsie. Tijdens haar studie- en promotieperiode is zij tevens actief geweest in het bestuur en commissies van de Groninger Wielrenvereniging Tandje Hoger. Anne Marijn heeft haar co-schappen achtereenvolgens gelopen in het UMCG en Nij Smellinghe te Drachten. Haar semi-arts stage heeft zij gedaan bij de afdeling Obstetrie en Gynaecologie en de afdeling Intensive Care van het Martini Ziekenhuis te Groningen.

Op 27 januari 2016 ontvangt zij haar artsenbul en mag zij haar proefschrift verdediging in het Academiegebouw te Groningen. Op 1 februari 2016 start Anne Marijn als ANIOS Obstetrie en Gynaecologie in het Martini Ziekenhuis te Groningen.

LIST OF PUBLICATIONS

Tsjitske J. Toering, **Anne Marijn van der Graaf**, Folkert W. Visser, Hendrik Buikema, Gerjan Navis, Marijke M. Faas* & A. Titia Lely*. Gender differences in response to acute and chronic angiotensin II infusion: a translational approach. *Physiological Reports* ISSN 2051-817X

Anne-Roos Sophie Frenay, Saleh Yazdani, Miriam Boersema, **Anne Marijn van der Graaf**, Femke Waanders, Jacob van den Born, Gerjan J Navis, Harry van Goor. Incomplete restoration of angiotensin II - induced renal damage despite complete functional recovery. *PLoS One*. 2015 Jun 10;10(6):e0129732. doi: 10.1371/journal.pone.0129732. eCollection 2015.

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van der Graaf AM*, Toering TJ*, Faas MM, Lely AT.From preeclampsia to renal disease: a role of angiogenic factors and the renin-angiotensin aldosterone system? *Nephrol Dial Transplant.* 2012 Oct;27 Suppl 3:iii51-7. doi: 10.1093/ndt/gfs278. Review.

van der Graaf AM, Wiegman MJ, Plösch T, Zeeman GG, van Buiten A, Henning RH, Buikema H, Faas MM. Endothelium-dependent relaxation and angiotensin II sensitivity in experimental preeclampsia. *PLoS One*. 2013 Nov 6;8(11):e79884. doi: 10.1371/journal.pone.0079884. eCollection 2013.

Anne Marijn van der Graaf, Gerda G. Zeeman, Henk Groen, Claire Roberts, Gus A. Dekker. Non-invasive assessment of maternal hemodynamics in early pregnancy. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health*, October 2013, Volume 3, Issue 4, Pages 261–269

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Plattel WJ, van den Berg A, Visser L, **van der Graaf AM**, Pruim J, Vos H, Hepkema B, Diepstra A, van Imhoff GW. Plasma thymus and activation-regulated chemokine as an early response marker in classical Hodgkin's lymphoma. *Haematologica*. 2012 Mar;97(3):410-5. doi: 10.3324/haematol. 2011.053199. Epub 2011 Nov 4.

de Leeuw K, Bijzet J, **van der Graaf AM**, Stegeman CA, Smit AJ, Kallenberg CG, Bijl M.Patients with Wegener's granulomatosis: a long-term follow-up study. *Clin Exp Rheumatol*. 2010 Jan-Feb;28(1 Suppl 57):18-23.

*these authors contributed equally to this work.